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(54) Title: NUCLEIC ACID SEQUENCES AND PROTEINS INVOLVED IN CELLULAR SENESCENCE

(57) Abstract: The present invention relates to nucleic acid sequences and proteins involved in senescence and particularly, to nucleic acid sequences and proteins including amphiphysin and caveolin involved in cellular senescence and their use.

NUCLEIC ACID SEQUENCES AND PROTEINS INVOLVED IN CELLULAR SENESCENCE

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to nucleic acid sequences and proteins involved in senescence and particularly, to nucleic acid sequences and proteins involved in cellular senescence and their use.

DESCRIPTION OF THE RELATED ART

The mechanism on senescence (also, called "aging") was intensively studied and a variety of hypotheses were suggested. The hypothesis comprises (a) free radical theory of aging (Harman D, *Proc. Natl. Acad. Sci.*, 78, 7124-7128(1981)), (b) crosslinking theory of aging (Bjorksten J., *J. Am. Geriatr Soc.*, 16, 408-423(1968)), (c) mitochondrial theory of aging (Lee CM et al., *Free Radic. Biol. Med.*, 22, 1259-1269(1997); and Wallace DC et al., *Biofactors*, 7, 187-190(1998)), and (d) genetic program theory of aging (Harley CB et al., *Curr. Opin. Genet. Dev.*, 5, 249-255(1995)).

Moreover, the senescence has been investigated in cellular level, i.e., cellular senescence. According to the investigation, senescent cell is characterized by (a) arrest of cell cycle at G1 phase, (b) diminished

physiological functions (Goldstein, *Science*, 249:1129-1133(1990); Campisi J., *Cell*, 84:497-500(1996)), and (c) resistance to apoptotic-programmed-cell death (Wang E., *Cancer Res.*, 55:2284-2292(1995)).

A large variety of studies on cellular senescence have been made with human fibroblasts since the cells are considered to reflect a senescence phenomenon in individual level (Campisi J., *Cell*, 84:497-500(1996)).

Meanwhile, the patent applications related to nucleic acid and proteins associated with aging process, disclosed in WO 99/52929 and WO 01/23615.

As described above, a variety of theories have been proposed, there remains a need of more evident elucidation for cellular senescence, a need of specific biomarker for identifying senescent cell, and a need of biomolecule for modulating cellular senescence.

In particular, the prospect of reversing senescence and restoring normal physiological function has an importance in certain diseases associated with senescence, for example, Werner Syndrome and Hutchinson-Gilford Syndrome.

Throughout this application, various patents and publications are referenced and citations are provided in parentheses. The disclosure of these patents and publications in their entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which

this invention pertains.

SUMMARY OF THE INVENTION

In one aspect of this invention, there is provided a method for detecting a senescent cell comprising determining the amount of a protein involved in cellular senescence of a cell, wherein the protein is one or more selected from the group consisting of amphiphysin protein and caveolin protein.

In another aspect of this invention, there is provided a method for detecting a senescent cell comprising determining the amount of a polynucleotide encoding a protein involved in cellular senescence of a cell, wherein the protein is one or more selected from the group consisting of amphiphysin protein and caveolin protein.

In still another aspect of this invention, there is provided a composition for modulating cellular senescence comprising the effective amount of a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

In further aspect of this invention, there is provided a composition for modulating cellular senescence comprising the effective amount of a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

In still further aspect of this invention, there is provided a composition for modulating cellular senescence comprising the effective amount of an antisense oligonucleotide which hybridizes to a polynucleotide encoding a protein involved in cellular senescence and thereby inhibits the polynucleotide from expressing the protein, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

In another aspect of this invention, there is provided a composition for modulating cellular senescence comprising the effective amount of a methylating agent or a demethylating agent, in which the agent methylates or demethylates bases of a polynucleotide encoding caveolin protein.

In still another aspect of this invention, there is provided a composition for modulating cellular senescence comprising the effective amount of dominant negative amphiphysin-1 gene.

In further aspect of this invention, there is provided a method for modulating cellular senescence in a patient in need thereof, comprising administering to the patient the effective amount of a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

In still further aspect of this invention, there is provided a method for modulating cellular senescence in a

patient in need thereof, comprising administering to the patient the effective amount of a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

In another aspect of this invention, there is provided a method for modulating cellular senescence in a patient in need thereof, comprising administering to the patient the effective amount of an antisense oligonucleotide which hybridizes to a polynucleotide encoding a protein involved in cellular senescence and thereby inhibits the polynucleotide from expressing the protein, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

In still another aspect of this invention, there is provided a method for modulating cellular senescence in a patient in need thereof, comprising administering to the patient the effective amount of a methylating agent or a demethylating agent, in which the agent methylates or demethylates bases of a polynucleotide encoding caveolin protein.

In further aspect of this invention, there is provided a method for identifying a substance affecting the senescence of a cell, which comprises: (a) culturing the cell in the presence of the substance to be tested; (b) isolating a protein from the cell; (c) contacting the isolated protein with an antibody specific to a protein

involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein; and (d) determining the amount of the isolated protein bound to the antibody.

In still further aspect of this invention, there is provided a method for identifying a substance affecting the senescence of a cell, which comprises: (a) culturing the cell in the presence of the substance to be tested; (b) isolating RNA from the cell; (c) contacting the isolated RNA with a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein; and (d) determining the amount of the isolated RNA hybridized to the polynucleotide encoding a protein involved in endocytosis.

In another aspect of this invention, there is provided a kit for detecting a senescent cell comprising a probe derived from a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

In still another aspect of this invention, there is provided a biomarker for identifying cellular senescence comprising a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

In further aspect of this invention, there is provided

a biomarker for identifying cellular senescence comprising a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

Accordingly, it is an object of this invention to provide a method for detecting a senescent cell.

It is another object of this invention to provide a composition for modulating cellular senescence.

It is still another object of this invention to provide a method for modulating cellular senescence in a patient in need thereof.

It is further object of this invention to provide a method for identifying a substance affecting the senescence of a cell.

It is still further object of this invention to provide a kit for detecting a senescent cell.

It is another object of this invention to provide a biomarker for identifying cellular senescence.

Other objects and advantages of the present invention will become apparent from the detailed description to follow taken in conjugation with the appended claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a confocal microphotograph representing reduced endocytosis in senescent cell;

Fig. 2 is a confocal microphotograph representing the internalization of trasferrin with increase of trasferrin treatment time in old and young cells;

Fig. 3 is a confocal microphotograph showing pulse-chasing transferrin uptake;

Fig. 4 is a confocal microphotograph showing the internalization of transferrin in induced senescent cell by H_2O_2 or hydroxyurea;

Fig. 5 is a photograph showing the results of western blotting for analyzing the expression of proteins associated with cellular senescence;

Fig. 6 is a photograph showing the results of western blotting for analyzing the expression of proteins associated with cellular senescence in induced senescent cell by H_2O_2 or hydroxyurea;

Fig. 7 is a photograph showing the results of northern blotting for analyzing mRNA encoding proteins associated with cellular senescense;

Fig. 8 shows a genetic map of the vector carrying cDNA encoding human amphiphysin-1 used in Example VII;

Fig. 9 shows a genetic map of the expression vector carrying cDNA encoding human amphiphysin-1 constructed in Example VII;

Fig. 10 shows a photograph representing image observed with fluorescence microscope for analyzing amphiphysin-1 expression in microinjected cells;

Fig. 11 represents a genetic map of the expression

vector carrying dominant negative amphiphysin-1 gene;

Fig. 12 represents a confocal microphotograph demonstrating the suppressed endocytic function of young cells treated with dominant negative amphiphysin-1 gene;

Fig. 13a shows a photograph representing the results of western blotting for analyzing the activation (phosphorylation) of Erk-1/2 kinase in young and middle cells;

Fig. 13b shows a photograph representing the results of western blotting for analyzing the activation (phosphorylation) of Erk-1/2 kinase in old cells;

Fig. 14 shows a photograph representing the results of western blotting for analyzing the expression of caveolin subtypes, that is, caveolin-1, caveolin-2 and caveolin-3, in young, middle and old cells;

Fig. 15 shows a photograph representing the results of immunoprecipitation, which verifies the interaction between epidermal growth factor receptor (EGFR) and caveolin-1 in young and old cells;

Fig. 16 is an electron microphotograph showing caveolae structure in young and old cells;

Fig. 17 represents a genetic map of the expression vector carrying caveolin-1 cDNA constructed in Example XV;

Fig. 18 represents a photograph showing the results of western blotting for young cells transformed with caveolin-1 cDNA;

Fig. 19 represents a confocal microphotograph

indicating that caveolin-1 expression is dramatically decreased by antisense oligonucleotide;

Fig. 20 is a photograph showing the results of western blotting for analyzing Erk-1/2 activation upon transfection of antisense oligonucleotie to caveolin-1;

Fig. 21 is a confocal microphotograph representing activation and localization of Erk-1/2 upon epidermal growth factor (EGF) stimulation in young and old cells;

Fig. 22 shows a confocal microphotograph representing activation and localization of Erk-1/2 upon EGF stimulation, in young and old cells, after downregulation of caveolin-1, that is after transfection of antisense oligonucleotide to caveolin-1;

Fig. 23a is a photograph representing the results of senescence-associated β-galactosidase activity staining for young cells treated with demethylating agent, 5-aza-deoxycytidine; and

Fig. 23b shows a photograph representing the results of western blotting for young cells treated with demethylating agent, 5-aza-deoxycytidine.

DETAILED DESCRIPTION OF THIS INVENTION

The present invention, in principle, is directed to nucleic acids and proteins modulating cellular senescence. The inventors have found that amphiphysin and caveolin are responsible for cell senescence in each different manner, as demonstrated in Example.

The process of receptor-mediated endocytosis via clathrin-coated vesicle is composed of several steps, which include recruitment of the clathrin coats and fission of the coated bud (Schmid, S.L. *Annu. Rev. Biochem.*, 66:511-548(1997)). After binding of ligand to the receptor, such as epidermal growth factor (hereinafter referred at as "EGF"), receptor tyrosine kinase phophorylates clathrin, which in turn can provide a binding site for Src-homology-3 (SH3) domain of amphiphysin (Slepnev, V.I. et al., *Science*, 281:821-824(1998); Wang, L.H. et al., *J. Biol. Chem.*, 270:10079-10083(1995); and Ramjaun, A.R. et al., *J. Neurochem.*, 70:2369-2376(1998)). Although its precise mechanism of action is not clear, amphiphysin-1 is thought to involve the recruitment and oligomerization at the neck of endocytotic buds (Schmid, S.L. *Annu. Rev. Biochem.*, 66:511-548(1997); and Takei, K. et al., *Nat. Cell Biol.*, 133-139(1999)). Amphiphysin-1 bridges the AP2/clathrin coat and dynamin-1 to make an endosomal vesicle (Slepnev, V.I. et al., *Science*, 281:821-824(1998); Shupliakov, O., et al., *Science*, 276:259-263(1997); McMahon, H.T. et al., *FEBS Lett.*, 413:319-322(1997); David, C., et al., *Proc. Natl. Acad. Sci.*, 93:331-335(1996); and Urrutia, R., et al., *Proc. Natl. Acad. Sci.*, 94:377-384(1997)). The carboxyl-terminal domain of amphiphysin recruits GTPase dynamin to pinch off the coated buds (David, C., et al.,

Proc. Natl. Acad. Sci., 93:331-335(1996); and Urrutia, R., et al., *Proc. Natl. Acad. Sci.*, 94:377-384(1997). Disruption of the interaction of amphiphysin with either dynamin or clathrin and AP-2 inhibits clathrin-mediated endocytosis (Slepnev, V.I. et al., *Science*, 281:821-824(1998); Shupliakov, O., et al., *Science*, 276:259-263(1997); and Wigge, P. et al., *Curr. Biol.*, 7:554-560(1997)). These findings indicate that amphiphysin may act as a regulated liner protein that couples clathrin-mediated budding of endocytotic vesicles to dynamin-mediated vesicle fission. Furthermore, it have been reported that amphiphysin has several subtypes and amphiphysin-2 also has a SH3 domain and has a binding-affinity to dynamin as amphiphysin-1.

Caveolae are vesicular invaginations of the plasma membrane with a diameter of 50-100 nm and are involved in endocytosis such as transcytosis and ptocytosis and signal transduction (Engelman, J.A. et al., FEBS Lett., 428:205(1998)). Caveolin, a 21-24 kDa integral membrane protein, is a principal structural component of caveolae membranes in vivo. The stable expression of caveolin-1 or -3 gene to the mammalian cells without caveolin induced the formation of caveolae structures (Lipardi, C. et al., *J. Cell Biol.*, 140:617(1998)). Caveolin has been found as several subtypes *in vivo*. Caveolin-1 is a key constituent of caveolae structures. Caveolin-2, is expressed

ubiquitously in most cell types, supposedly forming a hetero-oligomer in basolaterally localized caveolae (Scheiffele, P. et al., *J. Cell Biol.*, 140:795(1998)). It has been reported that the expression of caveolin-3 is restricted to striated muscle cells (Tang, Z. et al., J. Biol. Chem., 271:2255(1996)).

I. Method for Detecting a Senescent Cell and Method for Identifying a Substance Affecting Cellular Senescence

The present methods employ proteins involved in endocytosis such as amphiphysin protein and caveolin protein.

In the present method, the signal indicating cell senescence is detected either by measuring the decreased level of amphiphysin protein in cell or by measuring the increased level of caveolin protein in cell.

The term "senescence" is used herein to have the same meaning as "aging." The term "old cell" is used herein to have the same meaning as "senescent cell." Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. For example, the terms used herein may be found in Benjamin Lewin, *Genes VII*, published by Oxford University Press(2000); and Kendrew et al., *The Encyclopedia of Molecular Biology*, published by Blackwell

Science Ltd.(1994).

Accoriding to the preferred embodiment, the cell is derived from mammalian cell such as human cell.

Amphiphysin used in this invention may be selected from amphiphysin subtypes as described above. It is preferred that the amphiphysin protein is amphyamphiphysin-1, which has been known to a main subtype as mentioned previously. Furthermore, the caveolin protein used may be selected from caveolin-1, caveolin-2 and caveolin-3. It is preferred that the caveolin used is caveolin-1 protein, which has also been known to a main subtype as described previously.

In the present method which uses antibody against amphiphysin or caveolin, the antibody may be obtained as methods known to those skilled in the art (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988)). The antibody may be polyclonal or monoclonal antibody. Monoclonal antibody may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Pat. No. 4,196,265. The step of determining the amount of the isolated protein bound to the antibody may be performed according to methods known to those skilled in the art such as radioimmunoassay and enzyme-linked immunosorbent assay. These methods are generally based on the detection of a label or marker such as radioactive, fluorescent, biological or enzymatic tags or labels.

In a preferred embodiment of this method, the method is

conducted by western blotting method. The general procedure of western blottingmethod is disclosed in Peter B. Kaufma et al., *Molecular and Cellular Methods in Biology and Medicine*, 108-121, CRC Press. The western blotting for this invention, preferably, comprises the steps of (a) lysing a cell sample to be measured; (b) preparing a protein from the lysed cell; (c) denaturating the prepared protein in solution containing SDS and 2-mercaptoethanol; (d) performing SDS-polyacrylamide gel electrophoresis; (e) transferrin the protein on gel to nitrocellulose (hereinafter referred to as "NC") membrane; (f) reacting the protein on NC membrane with a primary antibody to amphiphysin or caveolin, advantageously, amphiphysin-1 or caveolin-1; (g) reacting the primary antibody with a secondary antibody conjugated to enzyme catalyzing colorimetric reaction; (h) inducing the colorimetric reaction by adding a substrate for the enzyme of (g); and (i) measuring the intensity of color developed by the enzyme (g).

Preferred enzyme for colorimetric reaction includes, but not limited to, alkaline phosphatase, β-galactosidase, and horse radish peroxidase. Where using alkaline phosphatase, bromochloroindolylphosphate (BCIP), nitro blue tetrazolium (NBT) and ECF may be used as a substrate; in the case of using horse radish peroxidase, chloronaphtol, aminoethylcarbazol, diaminobenzidine and luminol may be used as a substrate.

As described in Examples, the inventors have found that the level of amphiphysin in cell is dramatically decreased in senescent stage and vice versa for caveolin. Therefore, the present method may be carried out qualitatively. For example, the strength and the thickness of western blotting band for detecting amphiphysin may be found to be dramatically decreased to the extent capable of detecting visually; in the case of western blotting for detecting caveolin, the strength and the thickness of the resulting band may be found to be dramatically increased. Consequently, with comparing the western blotting band derived from senescent cell to one derived from young cell, the senescence can be easily detected.

Moreover, the present method may be carried out in a quantitative manner. For example, the bands resulted from western blotting may be transformed to quantitative data with densitometor. In a specific example for analyzing 40 μg protein, if the level of expression of amphiphysin-1 in tested cell is 45 times less than young cell, the tested cell may be considered senescent.

The present methods employ a polynucleotide coding for proteins involved in endocytosis such as amphiphysin protein and caveolin protein.

In this method, the signal indicating cell senescence is detected either by measuring the decreased level of a polynucleotide encoding amphiphysin protein in cell or by

measuring the increased level of a polynucleotide encoding caveolin protein in cell.

Accoriding to the preferred embodiment, the cell is derived from mammalian cell such as human cell. It is preferred that the polynucleotide is one conding for amphiphysin-1 or caveolin-1 protein. It is preferred that the polynucletide used in this method is gDNA (genomic DNA), cDNA and mRNA.

In a preferred embodiment of this method, the method may be conducted by northern blotting method. The general procedure of northern blotting method is disclosed in Peter B. Kaufma et al., *Molecular and Cellular Methods in Biology and Medicine*, 102-108, CRC Press. The northern blotting for this invention, preferably, comprises the steps of (a) preparing RNA from cell to be tested; (b) performing electrophoresis with the prepared RNA; (c) transferrin the RNA to nylon or NC membrane; (f) hybridizing the transferred RNA with radio-labeled oligonucleotide probe complementary to amphiphysin mRNA or caveolin mRNA, advantageously, amphiphysin-1 or caveolin-1; and (g) measuring the intensity of the resulting band.

As described in Examples, the inventors have revealed that the level of amphiphysin RNA in cell is dramatically decreased in senescent stage and vice versa for caveolin. Therefore, the present method may be carried out qualitatively. For example, the strength and the thickness of northern blotting band for detecting amphiphysin RNA

may be found to be dramatically decreased to the extent capable of detecting visually; in the case of northern blotting for detecting caveolin RNA, the strength and the thickness of the resulting band may be found to be dramatically increased. Consequently, with comparing the northern blotting band derived from senescent cell to one derived from young cell, the senescence can be conveniently detected.

Moreover, the present method may be carried out in a quantitative manner. For example, the bands resulted from northern blotting may be transformed to quantitative data with densitometor. In a specific example for analyzing 50 μg of amphiphysin-1 RNA,, if the strength of band for tested cell is 15 times less than young cell, the tested cell may be considered senescent.

II. Composition for Modulating Cellular Senescence

The composition for modulating cellular senescence of this invention comprises biomolecule capable of modulating cellular senescence. The biomolecule includes: (a) protein such as amphiphysin protein and caveolin protein; and (b) a polynucleotide such as one encoding amphiphysin protein or caveolin protein. In addition, the biomolecule includes antisense oligonucleotide capable of hybridizing to a polynucleotide encoding amphiphysin protein or caveolin

protein. Meanwhile, the composition of this invention comprises a methylating agent or a demethylating agent to methylate or demethylate bases of a polynucleotide encoding caveolin protein.

As described above, while it is conceivable that the proteins may be delivered directly, a preferred embodiment involves providing a polynucleotide encoding amphiphysin or caveolin.

According to the preferred embodiment, the cell is derived from mammalian cell such as human cell. It is preferred that the protein involve in endocytosis is amphiphysin-1 or caveolin-1 protein.

In the composition containing a polynucleotide, it is preferred that the polynucletide is gDNA or cDNA and is carried by expression vector for eucaryotic cell. The polynucleotide encoding amphiphysin-1 includes, preferably, nucleotide sequence coding for amino acids sequence represented by SEQ ID NO:2 and, more preferably, nucleotide sequence corresponding to nucleotides 111-2195 of nucleotide sequence represented by SEQ ID NO:1. The polynucleotide encoding caveolin-1 includes, preferably, nucleotide sequence coding for amino acids sequence represented by SEQ ID NO:4 and, more preferably, nucleotide sequence corresponding to nucleotides 26-559 of nucleotide sequence represented by SEQ ID NO:3.

The expression vector used in this invention expresses

foreign gene in eucaryotic host, preferably mammalian cell, more preferably human cell. The promoter in the expression vector may be derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., adenovirus late promoter; vaccinia virus 7.5K promoter). Further, it is also possible, and may be desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems. A number of viral based expression systems may be utilized, for example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). It is desired to incorporate into the transcriptional unit an appropriate polyadenylation site. The example of commercial vectors used for this invention includes pcDNA 3 (Invitrogen; containing cytomegalo virus promoter and polyadenylation signal), pSI (Promega; containing SV 40 promoter and polyadenylation signal), pCI (Promega; containing containing cytomegalo virus promoter and polyadenylation signal), and pREP7 (Invitrogen; RSV promoter and SV 40 polyadenylation signal).

Furthermore, in the composition containing a polynucleotide encoding amphiphysin or caveolin, the polynucleotide may be delivered using viral vectors designed for gene therapy. For example, the delivery

systems includes, but not limited to, (a) adenoviral vectors (Stratford-Perricaudet and Perricaudet, In: *Human Gene Transfer*, Eds., Cohen-Haguenauer and Boiron, Editions John Libbey Eurotest, France, 51-61(1991); and Stratford-Perricaudet et al., *Hum. Gene Ther.*, 1:241-256(1991)); (b) adeno-associated virus vectors (LaFace et al., *Virology*, 162:483-486(1998); Zhou et al., *Exp. Hematol.*, 21:928-933(1993); and Walsh et al., *J. Clin. Invest.*, 94:1440-1448(1994)); and (c) retroviral vectors such as engineered variant of the Moloney murine leukemia virus (Kasahara et al., Science, 266:1373-1376(1994)).

In the present composition containing antisense oligonucleotide, the antisense oligonucleotide may hybridize, under intracellular conditions, to target DNA or RNA. Targeting double-stranded DNA with an antisense oligonucleotide leads to triple-helix formation; targeting RNA leads to double-helix formation. Antisense oligonucleotide may be designed to bind to the promoter and other control regions, exons and introns of target gene. The antisense oligonucleotide used in this invention may be substantially complementary to target polynucleotide. That is, the antisense constuct may have some base mismatches to target gene. It is more preferred that the antisense oligonucleotide hybridizes to a polynucleotied encoding caveolin protein, advantageously, caveolin-1 protein. It is the most preferred that the

antisense oligonucleotide hybridizes to translational initiation region of caveolin-1 mRNA.

In the present invention containing a methylating agent or a demethylating agent, the agent regulates the level of methylation for bases of caveolin gene. As described in Example, caveolin gene with less methylation level exhibits greater expression level. In a preferred embodiment, the caveolin gene to be methlyated or demethylated is caveolin-1 gene. More preferably, the modified region is a promoter of caveolin-1 gene and the most preferably, CpG island from the promoter of caveolin-1 gene. Example of methylating agent used in this invention includes, but not limited to, methylazoxymethanol acetate, Temozolomide and N-methyl-N-nitrosourea. Non-limiting example of demethylating agent used in this invention includes 5-aza-deoxycytidine, 5-azacytidine, 6-azacytidine and 8-azaguanine.

As a composition for modulating cellular senescence, the present invention provides a composition comprising the effective amount of dominant negative amphiphysin-1 gene. The dominant negative amphiphysin-1 gene, which has been known to block the function of amphiphysin-1 (Shupliakov, O. et al., *Science*, 276:259(1997); and Wigge P., *Curr. Biol.*, 7:554(1997)). The treatment with dominant negative amphiphysin-1 gene leads to cellular senescence

as described in Example. According to a preferred embodiment, the dominant negative amphiphysin-1 gene is a polynucleotide encoding a polypeptide comprising the amino acid sequence 250 to 588 represented by SEQ ID NO:2.

III. Method for Modulating Cellular Senescence

In the method of this invention, the effective amount of biomolecule related to amphiphysin or caveolin is typically administered to a cell. In particular, a polynucleotide encoding amphiphysin or caveolin or antisense oligonucleotide thereof may be introduced *in vivo* or *ex vivo* in accordance with the following methods: (a) microinjection (Capecchi, M.R., *Cell*, 22:479(1980)); (b) calcium phosphate co-precipitation (Graham, F.L. et al., *Virology*, 52:456(1973)); (c) electroporation (Neumann, E. et al., *EMBO J.*, 1:841(1982)); (d) liposome-mediated transfection (Wong, T.K. et al., *Gene*, 10:87(1980)); (e) DEAE-dextran treatment (Gopal, *Mol. Cell Biol.*, 5:1188-1190(1985)); and (f) particle bombardment (Yang et al., *Proc. Natl. Acad. Sci.*, 87:9568-9572(1990)).

According to a preferred embodiment, the cell used is derived from mammalian cell, more preferred, human cell. Preferably, protein, polynucleotide and antisense oligonucleotide administered are related to amphiphysin-1 or caveolin-1. It is preferred that the polynucleotide administerd is gDNA or cDNA. More preferably, the

polynucleotide administered is carried on expression vector for eucaryotic cell. In a preferred embodiment of a method using antisense construct, the antisense oligonucleotide is substantially complementary to the gene encoding caveolin, more preferably, caveolin-1. The most preferable embodiment comprises the antisense oligonucleotide hybridizes to translational initiation region of caveolin-1 mRNA.

In the present method using a methylating agent or a demethylating agent, the agent regulates the level of methylation for bases of caveolin gene. In a preferred embodiment, the caveolin gene to be methlyated or demethylated is caveolin-1 gene. More preferably, the modified region is a promoter of caveolin-1 gene and the most preferably, CpG island from the promoter of caveolin-1 gene. Example of methylating agent used in this invention includes, but not limited to, methylazoxymethanol acetate, Temozolomide and N-methyl-N-nitrosourea. Non-limiting example of demethylating agent used in this invention includes 5-aza-deoxycytidine, 5-azacytidine, 6-azacytidine and 8-azaguanine.

The common descriptions of between I, II and III are abbreviated in order to avoid the complexity of this specification leading to undue multiplicity.

IV. Kits and Biomarkers

As indicated above, the present invention provides a kit for detecting a senescent cell. All the essential materials and reagents required for detecting a senescent cell may be assembled together in a kit. The probe used may be useful for hybridization to DNA or RNA isolated from a cell to be tested. Furthermore, the probe used may be primer primer for use in any molecular biology assay known to those of skill in the art such as PCR and RT-PCR. Also included may be enzymes suitable for amplification nucleic acids such as Taq polymerase, dNTP mixture and buffers to provide the necessary reaction mixture for amplification.

In a preferred embodiment, the probe is derived from the polynucleotide encoding amphiphysin-1 protein or caveolin-1 protein. The probe, preferably, is immobilized on a solid support. Solid supports suitable for use in the kit of this invention are known to those of skill of the art, which includes glasses, plastics, polymers, metals, metalloids, ceramics and organics. According to more preferred embodiment, this invention provides a kit comprising an array of probes derived from the polynucleotide encoding amphiphysin-1 protein or caveolin-1 protein. The general techniques for microarray containing solid supports have been disclosed in many publications such as WO 89/10977, U.S. Pat. Nos. 5,202,231,

5,002,867 and 5,143,854.

According to preferred embodiment of this invention, the kit further comprises a label for detecting the presence of the probe. The label allows detection of hybridization of between the probe and nucleotides isolated from sample to be tested. The most common label is radioactive material such as ^{3}H, ^{14}C and ^{32}P.

The present invention provides a biomarker for identifying cellular senescence. In preferred embodiments, the protein or the polynucleotie suitable for this invention, is derived from amphiphysin-1 or caveolin-1.

The following specific examples are intended to be illustrative of the invention and should not be construed as limiting the scope of the invention as defined by appended claims.

EXAMPLE I: Cell Culture

I-1: Culture for Human Foreskin Fibroblast

Foreskin fibroblast was isolated and cultured according to the method provided by Boyce and Ham (Boyce ST. and Ham RG., *J. Invest. Dermato.*, 81:33-40(1983)) as follows: First, foreskin was obtained from 7-year-old Korean male and was stripped to give pieces, after which the foreskin pieces was added to 10 ml of Hank's salt

solution (Gibco BRL) containing 0.25% collagenase. Following incubating for 90 min. at 37℃ in CO_2-controlled (5%) incubator, epithelium and dermis were separated from each other. To the separated dermis, 1 ml of trypsin solution (0.25%) was added and the resulting solution was added to 10 ml of DMEM (Dulbecco's modified Eagle medium: Sigma) containing 100 μg/ml of streptomycin and 100 units/ml of penicillin, followed by incubating for 10 min. at 37℃. The yielded foreskin fibroblast were washed with 10 ml of PBS and in DMEM (supplemented with 10% FBS and antibiotics) were serially passaged as follows: The incubator was maintained to the atmosphere of 5% CO_2 and the temperature of 37℃, DMEM was renewed once per 3 days and subconfluency (about 80-90%) was kept and subculture was performed at a 1:4 ratio. The cells, cultured with less than 25 population doublings, were considered presenescent cells (or young cells), which are highly proliferative, while cells with over 60 population doublings were defined as senescent cells, which showed delayed population doublings times (over 3 weeks).

I-2: Culture for Fetal Lung Fibroblast

Fetal lung fibroblast, IMR-90, was purchased from ATCC (CCL-186). The culture for IMR-90 was carried out in the same manner as the above.

EXAMPLE II: Induction of Cellular senescence

II-1: Induction of Cellular senescence with H_2O_2

The fibroblasts subcultured in Exmaple I-1, PDL of which are 16, were placed in culture plate and were kept to arrest cell cycle to G1 phase in incubator (37℃, 5% CO_2 and humidified) for a week. The cell cycle arrest was confirmed as follows: Following the fixation of the cells with cold ethanol, the cells were stained for 30 min. at room temperature using PI staining solution (containing 50 μg/ml of Rnase A and 50 μg/ml of propinium iodide in PBS). Thereafter, using FACS (fluorescence-activated cell sorter), the cell cycle was confirmed by observing DNA phenotype, i.e., 2n or 4n. The cells in G1 phase were showed 2n of DNA phenotype.

The fibroblasts arrested in G1 phase were treated with 400 μM H_2O_2 and then incubated for 3 hrs., followed by washing with 10 ml of PBS. Then, the cells were subcultured at a 1:4 ratio and under normal conditions for cell culure (37℃, 5% CO_2 and humidified), cell culture was continuously performed. Following 7 days after the treatment, the cells were determined in terms of senescence using senescence-associated β-galactosidase activity staining as described in Example III.

II-2: Induction of Cellular senescence with Hydroxyurea

The fibroblasts subcultured in Exmaple I-1, PDL of

which are 16, were placed in culture plate containing DMEM and were cultured in incubator (37℃, 5% CO_2 and humidified) for 14 hrs. Thereafter, the fibroblasts were treated with 400 μM Hydroxyurea and then incubated continuously. The medium was renewed once per 3 days with the addition of fresh 400 μM Hydroxyurea. Following 14 days after the treatment, the cells were determined in terms of senescence using senescence-associated β-galactosidase activity staining as described in Example III.

Example III: Senescence-Associated β-Galactosidase Activity Staining

A senescence-associated β-galactosidase activity staining (hereinafter referred to as "SA β-gal activity staining)was performed according to the method of Dimri et al. (Dimri GP et al., *Proc. Natl. Acad. Sci.*, 92:9363(1995)): The semiconfluent fibroblasts were washed twice with 10 ml of PBS and fixed with 2% paraformaldehyde in PBS for 5 min. at room temperature. After washing with PBS, cells were incubated with SA β-gal activity staining solution (1 mg/ml of X-gal, 40 mM citric acid/sodium phosphate buffer, pH 6.0, 5 mM potassium ferrocyanide/ferricyanide, 150 mM NaCl and 2 mM $MgCl_2$) at 37℃ for 4 hrs. Young and old human fibroblasts were observed with phase contrast microscopy. As a result of the observation, human fibroblast showed β-galactosidase

activity from PDL 50, IMR 90 from PDL 65, cells treated with H_2O_2 from 10 days after treatment and cells treated with hydroxyurea from 14-20 days after treatment, which demonstrate the entry of cellular senescence.

Example IV: Evaluation of Alteration of Endocytosis

IV-1: Observation of Reduced Endocytosis in Senescent Cell

To investigate the functional changes of receptor-mediated endocytosis in senescent cell, the internalization of transferrin was observed. The fibroblasts were plated onto cover glasses and incubated in incubator (37℃, 5% CO_2 and humidified), followed by the treatment of 25 μg/ml tetramethylrhodamine-conjugated human transferrin (Molecular Probes) for 5 min. After washing 10 ml of PBS, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then, nuclei of cells were stained with DAPI (Sigma Aldrich). Internalization of fluorescent transferrin was monitored with confocal microscopy (Biorad, #MRC1024), which is shown in Fig. 1. In Fig. 1, panel A represents young (PDL 20) fibroblasts and senescent fibroblasts (PDL 54) and panel B represents IMR cells (PDL 32 and PDL 68, respectively). As demonstrated in Fig. 1, young fibroblasts and IMR cells took up fluorescent transferrin readily and internalized transferrin was observed as typical punctuated crescent shapes in the perinuclear area. In contrast, senescent cells did not uptake transferrin as

efficiently as presenescent cells.

IV-2: Observation of Internalization of Transferrin with Increase of Treatment Time

The fibroblasts were treated with 25 μg/ml tetramethylrhodamine-conjugated human transferrin for 10, 20, 40 or 60 min as described previously; and the experimental results are shown in Fig. 2. In Fig. 2, panels Y, M and O represent young fibroblasts (PDL 24), middle fibroblasts (PDL 38) and senescent fibroblasts (PDL 54), respectively. As shown in Fig. 2, cells with PDL 24 and PDL 38 took up fluorescent transferrin efficiently and internalized transferrin was observed in the perinuclear area in 10-min treatment, which was increased with the increase of treatment time, thereby giving greater fluorescence intensity. In contrary to this, the senescent fibroblasts did not uptake transferrin even after 60-min treatment.

IV-3: Pulse-Chasing of Transferrin Uptake

Twenty five μg/ml rhodamine-conjugated transferrin was pulsed on IMR 90 cells for 5 min and then chased for 0, 5 and 10 min. After fixing as described above, internalization of fluorescent transferrin was monitored with confocal microscopy (see Fig. 3). In Fig. 3, panels Y, M and O represent young (PDL 26), middle (PDL 48) and senescent cells (PDL 72), respectively. As shown in Fig. 3,

young cells efficiently uptake transferrin and localize it to perinuclear area just in 5 min and then the transferrin was quickly degraded after 10 min chasing. PDL 48 cells revealed a delayed and limited uptake of transferrin after 10 min chasing. PDL 72 cells nearly failed to uptake transferrin with the lapse of chasing time.

IV-4: Observation of Reduced Endocytosis in Artificially Induced Senescent Cell

The senescent cells which were artificially induced in Exampl II were treated with 25 μg/ml rhodamine-conjugated transferrin as above and internalization of fluorescent transferrin was monitored with confocal microscopy (see Fig. 4). As indicated in Fig. 4, not only naturally-occurring senescent cells through serial passage but also artificially-induced senescent cells by H_2O_2 or hydroxyurea (marked "+") showed the reduced function of receptor-mediated endocytosis.

In summary, the inventors have revealed that senescent fibroblast cells show significantly reduced function of endocytosis and thus fail to uptake a variety of ligands such as transferrin.

Example V: Analysis of Expression of Proteins Involved in Receptor-Mediated Endocytosis

To identify the molecular mechanism for such alteration in the receptor-mediated endocytosis of senescent cells, the expression level of several proteins involved in receptor-mediated endocytosis was checked through western blotting experiment.

First, total cell lysates were extracted from subconfluent early, middle and late-passaged cells using lysis buffer (1% Triton X-100, 0.5% NP-40, 50 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM $NaVO_4$ and 1 mM NaF) and sonicated briefly, after which the lysates were centrifuged at 14000 x g for 10 min and the supernatants were taken. With the supernatants, the protein quantification was performed as Bradford method (Bradford, M., *Anal. Biochem.* 72:248-254(1976)) and 40 μg of protein equivalents were boiled for 5 min in 5x SDS sample buffer (60 mM Tris-Cl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue). Cell lysates (10-15 μg of protein equivalents) were electrophoresd on 8% polyacrylamide gel using electrophoresis kit (Biorad) and then transferred to nitrocellulose membranes using transfer kit (Biorad). The blots were blocked with TTBS (Tris buffered saline with Tween 20) containing 5% non-fat dry milk (Difco) for 1 hr at room temperature. The blots were immunoblotted with the respective primary antibody in TTBS with 5% non-fat dry milk for 1 hr. at room temperature, washed three times with TTBS, and incubated with horseradish peroxidase-

conjugated anti-mouse secondary antibody (Jackson Immuno Research Laboratory). In the primary antibodies, anti-dynamin antibody, anti-α-adaptin, anti-β-adaptin and anti-clathrin heavy chain antibody were purchased from Transduction Laboratories, monoclonal antibody to amphiphysin-1 was prepared by Dr. Kim from Chungbuk National University (Jin, Y., Kim et al. (In press), Production and characterization of monoclonal antibodies against amphiphysin, *Exp. Mol. Med.*), and anti-phosphotyrosin antibody and anti-transferrin receptor antibody were Santa Cruz Biotechnology. The signals were finally visualized by an enhanced chemiluminescence system (ECL kit, Amersham Pharmacia Biotech), which are found in Fig. 5.

As demonstrated in Fig. 5, only amphiphysin-1, but none of other endocytotic protein tested, was significantly reduced in senescent cells. The unique reduction in expression of amphiphysin-1 protein was observed in the senescent cells of both foreskin fibroblasts and IMR 90 cells. Moreover, the senescent cells which were induced by H_2O_2 or hydroxyurea (marked "+") gave the same results as shown in Fig. 5 (see Fig. 6).

These results indicate that the cellular senescence process is accompanied with down regulation of amphiphysin-1.

Example VI: Northern Analysis of mRNA Encoding Proteins

Involved in Receptor-Mediated Endocytosis

To investigate the exact mechanism for the reduced level of amphiphysin-1 protein in senescent cells, which was analyses in Example V, northern blotting was carried out as follows:

The RNA was isolated from human fibroblasts of Example I using acid guanidinium thiocyanate-phenol-chloroform, mixed with formaldehyde sample buffer (5x MOPS, 17.5% formaldehyde and 50% formamide), and then electrophoresed on 1% agarose gel using electrophoresis kit (Hoefer). Following the electrophoresis, the RNA was transferred to nitrocellulose membrane and was cross-linked to the membrane using auto UV crosslinker (Stratagen). Then, the hybridization was performed with p^{32}-labelled probe (comprising bases 111-1116 of amphiphysin-1 cDNA) and the resulting autoradiograms were obtained (see Fig. 7). In Fig. 7, panels Y, M and O represent PDL 27, PDL 36 and PDL 60 fibroblasts, respectively. According to Fig. 7, it was elucidated that the level of amphiphysin mRNA was reduced with a progression of senescence.

Theses results indicate that the cellular senescence process is accompanied with down regulation of amphiphysin-1 at transcriptional level.

Example VII: Cloning of Amphiphysin-1 Gene

The cDNA encoding the full length of human amphiphysin-1 has a nucleotide sequence represented by SEQ ID NO:1 and the vector carrying the cDNA was obtained from Chungbuk National University (Korea). The vector was constructed in such a manner that the cDNA was inserted between BamHI and EcoRI restriction sites of pGEX-2T vector (Pharmacia) and thereby amphiphysin-1 and glutathione-S-transferase were expressed in fused form. Fig. 8 shows genetic map of the final vector carrying cDNA encoding human amphiphysin-1. The full length cDNA was amplified by PCR using a set of primers: 5′-AACTGTCCACCATGGCCGACATCAAGACGGGC-3′ and 5′-GGATCCCTAATCTAAGCGTCGGGT-3′. The PCR amplification was performed for 30 cycles using Pyrobest Taq polymerase (Takara) in accordance with following temperature sets: 55℃ for 30 sec (annealing), 72℃ for 1.5 min (extension) and 92℃ for 30 sec (denaturation). The amplified cDNA was cloned into pT7 blue vector (Novagen) and its base sequence was determined. After digestion with Hin*dIII* and Bam*HI*, the cDNA was subcloned into pcDNA3 vector (Invitrogen: containing promoter and polyadenylation signal of cytomegalo virus) in order to microinject the amplified cDNA of amphiphysin-1 to fibroblast. The pcDNA3 containing cDNA of amphiphysin-1 was showed in the genetic map of Fig. 9.

Example VIII: Restoration of Endocytic Function in Senescent Cell by Amphiphysin-1 Gene

VIII-1: Microinjection of Amphiphysin-1 Gene

Senescent fibroblasts (PDL 58) in Example I were placed onto cover glass and incubated for 24 hrs. in DMEM with on FBS in incubator (37℃, 5% CO_2 and humidified). Then, 10^{-14} ℓ of amphiphysin-1 gene cloned into pcDNA3 of Example VII (10 ng/ml) and 10^{-14} ℓ of rabbit IgG (Sigma, 5 mg/ml) were microinjected into nucleus of senescent fibroblast. The vector was diluted to 10 ng/ml in the microinjection buffer (50 mM HEPES, pH 7.2, 100 mM KCl, 50 mM $NaPO_4$). The diluted vector was microinjected into nucleus using transjector 5426 (Eppendorf) and micromanipulator (Eppendorf).

VIII-2: Analysis of Amphiphysin-1 Expression in Microinjected Cell

Using double immunofluorescent staining method, the expression of amphiphysin-1 in microinjected cell was analyzed. Following 24 hrs. incubation in DMEM without FBS after microinjection, the cells were fixed with 3.7% paraformaldehyde (in PBS) for 10 min. and then permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature. The cells were sequentially incubated with anti-amphiphysin-1 antibody, FITC-conjugated anti-rat

IgG antibody (Jackson Laboratory: 1:100 dilution) for 1 hr at 37℃, and then rhodamine-conjugated anti-rabbit IgG antibody (Jackson Laboratory: 1:100 dilution) for 1 hr at 37℃. The image was observed with fluorescence microscope (Zeiss, Axiovert25, CFL451210) (see Fig. 10). As shown in Fig. 10, anti-rabbit IgG gives red fluorescence emitted by rhodamine and amphiphysin-1 is determined by green fluorescence emitted by FITC of secondary antibody. The green fluorescence demonstrating the existence of amphiphysin-1 was observed in cytoplasm.

Therefore, it is elucidated that amphiphysin-1 protein is expressed in the microinjected cells in Example VIII-1.

VIII-3: Analysis of Restoration of Endocytic Function in Senescent Cell

Microinjected cells were incubated for 24 hrs. in DMEM without FBS in incubator (37℃, 5% CO_2 and humidified). Then, the cells were treated with 125 ng/ml tetramethylrhodamine-conjugated transferrin for 30 min., washed with 10 ml of PBS and fixed with 10 ml of 4% formaldehyde (in PBS) for 10 min. The immunofluorescence staining was performed as described in Example VIII-2. By means of fluorescence microscope (Zeiss, Axiovert 100) microinjected cells and uptaken transferrin were analyzed. The results are summarized in Table 1.

Table 1

DNA[1)]	Ab[2)]	Microinjected Cell			Non-Microinjected Cell		
		Tf[3)]	Total[4)]	Ave[5)] (%)	Tf	Total	Ave (%)
PcDNA3	Anti-rabbit IgG Ab	1	12	7.50	4	32	11.09
		1	15		3	31	
Ap-1[6)] gene + pcDNA3	Anti-rabbit IgG Ab	1	12	7.50	18	46	36.81
		1	15		10	29	
	Anti-amphiphysin-1 Ab	1	12	10.42	18	37	48.65
		2	16				

[1)]microinjected DNA; [2)]antibody for analyzing microinjected cell; [3)]transferrin; [4)]total cell number; [5)]average value; and [6)]amphiphysin-1.

As known in Table 1, compared to cells microinjected with pcDNA3 as mock, the microinjected cells with pcDNA3 carrying amphiphysin-1 gene shows much higher transferrin-uptake activity. In other words, the endocytic activity of senescent cells was sharply increased by the introduction of amphiphysin-1 cDNA. These results successfully demonstrate that amphiphysin-1 is essential for the restoration of functional endocytosis of the senescent cells and thus is essential for modulating

cellular senescence.

Example IX: Cloning of Dominant Negative Amphiphysin-1 Gene

Dominant negative amphiphysin-1 gene, which is known to block the function of amphiphysin-1 (Shupliakov, O. et al., *Science*, 276:259(1997); and Wigge P., *Curr. Biol.*, 7:554(1997)), was amplified by PCT using amphiphysin-1 cDNA as template and specific primers, thereby amplifying a partial nucleotide sequence encoding the middle part of amphiphysin-1 protein (amino acids 250 to 588). The forward primer and reverse primer used have the following sequences: 5′-AACTGTCCACCATGAGTGATTCGGGTCCTCTCCGC-3′ and 5′-GGATCCCTACTGCTCCGTAGCCAGCTCCGG-3′, respectively. The PCR amplification was performed and the amplified product was subcloned using pcDNA 3 (Invitrogen) in the same manner as Example VII. The genetic map of the final vector is shown in Fig. 11.

Example X: Suppression of Endocytic Function in Young Cell by Dominant Negative Amphiphysin-1 Gene

Using the vector constructed in Example IX, young fibroblasts (PDL 16) were transformed as follows: Two μg of the vector constructed in Example IX and 0.5 μg of pEGFP-N1 vector (Clontech) were mixed with DMEM and 8 μl of Plus

reagent, after which the resultant was allowed to stand for 15 min. at room temperature. Thereafter, the mixture was mixed well with Lipofectamine (Gibco-BRL) and DMEM, followed by standing the mixture for 15 min. at room temperature. Following the further addition of 2 ml of DMEM, the final mixture was added to young fibroblasts and then incubated for 3 hrs at 37℃. After the lapse of 3 hr., 2.5 ml of DMEM containing 20% FBS were added and incubated for another 40 hr. The incubated cells were treated with 25 μg/ml rhodamine-conjugated transferrin for 10 min. and the image was observed by confocal microscope (Biorad, #MRC1024), thereby elucidating the internalization of transferrin either in transformed cell (emitting EGFP-derived green fluorescence) or in non-transformed cell (see Fig. 12). As shown in Fig. 12, co-transformed cells with the vector carrying dominant negative amphiphysin-1 cDNA and pEGFP-N1 (left panel) show no red fluorescence by rhodamine-conjugated transferrin, indicating that the internalization of rhodamine-conjugated tansferrin does not occur, but non-transformed cells (right panel) show red fluorescence.

These results demonstrate that the functional incompetence of amphiphysin-1 can inhibit receptor-mediated endocytosis and finally induce cellular senescence.

Example XI: Analysis of Erk-1/2 Activation by Western

Blotting

Young cells (PDL less than 30), middle cells (PDL 35-45) and old cells (PDL more than 60) of Human fibroblasts or IMR-90 cells, respectively were stimulated with 100 ng/ml EGF (Gibco-BRL, human, recombinant). After stimulation, western blotting was performed as described in Example V. Monoclonal anti-phospho-Erk-1/2 antibody, polyclonal anti-Erk-1/2 antibody and polyclonal anti-EGFR antibody were purchased from Santa Cruz Biotechnology, Inc. As found in Fig. 13a, in both young and middle-aged cells, Erk-1/2 kinases were phosphorylated (activated) within 5 min, and the resulting activation was sustained for 15 min after EGF stimulation. However, the phosphorylation of Erk-1/2 kinase from old cells was not detected until 15 min had lapsed. The expression level of Erk kinase and and EGFR was not changed by the increase of population doubling in Western blot (see Fig. 13b), despite the down-regulation of EGF signaling to Erk kinases.

Therefore, the reduced responsiveness of old cells to growth factor is due to the reduced Erk-1/2 phosphorylation, i.e., the reduced Erk-1/2 activation.

Example XII: Analysis of Caveolin Expression by Western Blotting

Analysis of caveolin expression, in young cells (PDL

less than 30), middle cells (PDL 35-45) and old cells (PDL more than 60) of Human fibroblasts or IMR-90 cells, was performed by Western blotting as described in Example V. Monoclonal anti-caveolin-1 antibody, monoclonal anti-caveolin-2 antibody and monoclonal anti-caveolin-3 antibody were purchased from Transduction Laboratories. As shown in Fig. 14, with aging, all of caveolin-1, caveolin-2 and caveolin-3 were expressed increasingly in both human fibroblasts and IMR-90 cells.

Example XIII: Analysis of Interaction between EGFR and Caveolin-1 by Immunoprecipitation

Young (PDL less than 30) or old (PDL more than 60) fibroblasts were lysed in IP lysis buffer (10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg /ml leupeptin, 50 mM NaF, 0.2 mM Na_3VO_4) and sonicated briefly. Lysates were spin down at 9,000 rpm for 5 min., and supernatants were incubated with normal mouse serum, anti-EGFR antibody or anti-caveolin-1 antibody. Immune complexes were precipitated with protein A-Sepharose beads (Amersham Pharmacia Biotech) and separated by SDS-polyarylamide gel electrophoresis and analyzed by Western blot as described in Example V (see Fig. 15).

As demonstrated in Fig. 15, the immune complex of EGFR from old fibroblasts contained caveolin-1 proteins,

while young fibroblasts did not show a comparable amount of caveolin-1 and subsequent interactions with EGFR.

It is elucidated that with aging, the expression of caveolin-1 protein is increased and then the increased caveolin-1 protein is interacted with EGFR to inhibit the activation of Erk-1/2 kinase by EGFR.

Example XIV: Electron Microscopic Analysis for Caveolin-1

Subconfluent young (PDL 20) and old (PDL 65) fibroblasts were palletized by centrifugation (1,000 rpm) and fixed with 3% glutaraldehyde/phosphate-buffered saline at pH 7.4. After washing with 0.2 M sodium cacodylate buffer, pH 7.4, cell pellets were treated with 1% osmium tetroxide in cacodylate buffer for 1 hr. The cells were then dehydrated in graded ethanol steps through propylene oxide and embedded in Embed812 (Electron Microscope Sciences). The embedded cells were cut to the size of 200 nm by microtomb (Leichert-JUNG) and the cuts were stained with methylene blue and azure II, followed by observation with light microscopy in order to select the appropriate observation region of electron microscopy. Thereafter, the selected region was ultra-cut to the size of 60 nm by microtomb (Leichert-JUNG) and stained with uranyl acetate and lead citrate. Sections were observed using a transmission electron microscope (H-600, Hitachi). Fig. 16 indicates that old fibroblasts contain significantly more

caveolae-like structure than young cells.

Example XV: Construction of Plasmid DNA Carrying Caveolin-1 cDNA

Total RNA was isolated from human old fibroblasts using TRIzol (Gibco-BRL, #15596-026) and RT-PCR was then carried out to obtain caveolin-1 cDNA using the isolated RNA. The primers used are: forward primer, 5′-atccaagcttccaccatgtctgggggcaaatacgt-3′ and reverse primer, 5′-gcaggatccctatatttctttctgcaagttgat-3′. The reverse transcriptase and Taq polymerase used are AMT RTase from Promega and Ex Taq from TaKaRa, respectively. The temperature is set: 60℃ for 30 sec (annealing), 72℃ for 50 sec (extension) and 92℃ for 30 sec (denaturation). The amplified cDNA was verified by DNA sequencing in accordance with chain termination method (Sanger, F. et al., *Proc. Natl. Acad. Sci.*, 74:5463(1977)). The nucleotide sequence of caveolin-1 cDNA is found in SEQ ID NO:3. The amplified cDNA was subcloned into pcDNA3 (Invitrogen) using restriction sites of Hin*dIII* and Bam*HI*. The genetic map of the final constructed vector is shown in Fig. 17.

Example XVI: Transformation of Human Fibroblasts with Caveolin-1 cDNA

Young human fibroblasts (PDL 25) were transformed using the vector constructed in Example XV. The cells were plated into the dish and incubated for 18 hrs to allow 70-80% confluency. Two μg of the vector constructed in Example XV was mixed with 8 μl of Plus reagent and the resulting mixture was mixed with 12 μl of Lipofectamine (Gibco-BRL) and 238 μl of DMEM, followed by standing the mixture for 15 min. at room temperature. Following the further addition of 2 ml of DMEM, the final mixture was added to young fibroblasts and then incubated for 3 hrs at 37℃. After the lapse of 3 hr., 2.5 ml of DMEM containing 20% FBS were added and incubated for another 24 hr. After 30 hr, the transfected cells were stimulated with 100 μg/ml EGF. Finally, Western blotting was carried out as described in Example V so that the activation of Erk-1/2 may be detected. In Fig. 18, lane 1 of panel A represents sample transformed with pcDNA 3 and lane 2 represents sample transformed with pcDNA 3 carrying caveolin-1 cDNA. As verified in Fig. 18, the cells transformed with pcDNA 3 carrying caveolin-1 cDNA express caveolin-1 protein. In Fig. 18, lanes 1-3 of panel B represent samples transformed with pcDNA 3 and subsequently stimulated with EGF for 0, 5 and 20 min, respectively, and lanes 4-6 of panel B represent samples transformed with pcDNA 3 carrying caveolin-1 cDNA and subsequently stimulated with EGF for 0, 5 and 20 min.

As shown in Fig. 18, the expression of Erk-1/2

kinase was not changed in cells overexpressing caveolin-1 protein whereas the phosphorylation of Erk-1/2 was significantly inhibited in comparison with mock-transformed cells.

These data demonstrate that the activation of Erk-1/2 kinase is blocked when introducing caveolin-1 DNA into cells and sequentially the responsiveness to stimuli is diminished, thereby leading to cellular senescence. The result reveals that the diminished activation of Erk-1/2 kinase is due to the diminished expression level of caveolin-1 protein.

Example XVII: Transfection of Antisense Oligonucleotide to Caveolin-1

XVII-1: Synthesis of Antisense Oligonucleotide to Caveolin-1

To prepare antisense oligonucleotide to inhibit the expression of caveoin-1 protein, a suitable region of caveolin-1 mRNA to interact with antisense oligonucleotide was selected. Thus, the antisense oligonucleotides capable of binding to translational initiation region of caveolin-1 mRNA, which are designed to block translational initiation, were synthesized. The synthesized oligonucleoties were conjugated with fluorescien (Genotech) in their 5'-terminal region and modified by phosphorothioate to increase their stability. For example,

the synthesized antisense oligonucleotide has a nucleotide sequence: 5′-tttgccccccaga-3′. The sense oligonucleotide bound to the above region was also synthesized: 5′-atgtctgggggc-3′.

XVII-2: Transfection of Antisense Oligonucleotide to Caveolin-1

Old human fibroblasts (PDL 64) were placed onto 24 well-plate or dish containing DMEM without FBS and incubated in incubator (37℃, 5% CO_2) for 12 hr. After incubation, 1-5 ㎕ of 100 μM antisense oligonucleotides synthesized and Plus reagent (Gibco-BRL) were mixed with 500 ㎕ of DMEM and subsequently reacted for 15 min. at room temperature, and the resulting mixture was well mixed with 500 ㎕ of the mixture containing 12 ㎕ of Lipofectamine (Gibco-BRL) and DMEM, followed by standing the mixture for 15 min. at room temperature in order to form liposome complex. The incubated cells were washed twice with DMEM (no serum) and treated with 1 ml of the liposome complex, followed by incubation for 3 hr at 37℃. To the transfected cells, 1-5 ml of DMEM containing 10% FBS was added and the incubation was subsequently carried out for 24 hr, after which the media was changed with DMEM containing 10% FBS. Following incubation for a given period, immunostaining and western blotting were carried out as follows:

XVII-3: Immunostaining

The cells treated with antisense oligonucleotide were fixed with 0.5 ml of 4% paraformaldehyde for 20 min at room temperature and then permeabilized with 0.5 ml of 0.5% Triton X-100 in PBS for 10 min, followed by blocking with 2% BSA (in PBS). The cells were sequentially incubated with anti-caveolin-1 antibody (Transduction Laboratory) overnight at 4℃ and then rhodamine-conjugated secondary antibody (Santa Cruz) for 1 hr at room temperature. For the purpose of visualizing nucleus, DAPI (Molecular Probe) was also added. The observation was performed using confocal microscope (Biorad, #MRC1024). As shown in Fig. 19, the expression of caveolin-1 in cell is dramatically decreased in the cells treated with antisense oligonucleotide with a lapse of treatment time, while the expression of caveolin-1 is not changed in the cells treated with sense oligonucleotide. Interestingly, the old cells treated with antisense oligonucleotide exhibited the altered cell morphology: enlarged and spread morphology to smaller and spindle morphology. In contrast, the old cells treated with sense oligonucleotide did not show such cell-morphology alteration.

XVII-4: Western Blotting

Western blotting was performed with the cells treated with oligonucleotides in the same manner as Example XII. The cells treated with antisense

oligonucleotide provided a weaker band corresponding to caveolin-1, indicating that the expression of caveolin-1 is decreased in the cells treated with antisense oligonucleotide.

Based on the results from immunostaining and western blotting, it is elucidated that caveolin-1 is directly involved in cellular senescence and the inhibition of expression of caveolin-1 leads to not only the prevention of cellular senescence but also the conversion of old cell to young cell.

Example XVIII: Analysis of Erk-1/2 Activation Upon Transfection of Antisense Oligonucleotide to Caveolin-1

The old and young fibroblasts were treated with antisense oligonucleotide as described in Example XVII. The EGF stimulation and western blotting were carried out as described in Example XI. Fig. 20 represents the results of this Example. As shown in Fig. 20, Erk-1/2 kinases in young cells were strongly phosphorylated (activated). However, non-treated old cells and treated old cells with sense oligonucleotide showed higher basal Erk-1/2 activity than young cells and when stimulated with EGF, the cells showed no alteration in Erk-1/2 activation. Interestingly, in old cells treated with antisense oligonucleotide, Erk-1/2 activation by EGF was highly increased as young cells.

These observations elucidates that the inhibition of caveolin-1 expression due to the treatment with antisense oligonucleotide, provide old cells with the restoration of signal cascade mediated by Erk, which is typical in young cells.

Example XIX: Observation of p-Erk-1/2 Translocation to Nucleus Upon Transfection of Antisense Oligonucleotide to Caveolin-1

To verify that Erk-1/2 kinase activated in Example XVIII is translocated into nucleus and regulate sequentially the expression of other genes, immnostaining was performed as follows: Young and old fibroblasts were treated with antisense oligonucleotide and EGF as Example XIII. The treated cells were sequentially incubated with anti-p-Erk (phosphorylated-Erk) antibody (New England Biotech) overnight at 4℃ and then FITC-conjugated secondary antibody (Santa Cruz) for 1 hr at room temperature. For the purpose of visualizing nucleus, DAPI (Molecular Probe) was also added. The image of p-Erk-1/2 localization was visualized using confocal microscope (Biorad, #MRC1024), which is found in Figs. 21 and 22. In Figs. 21 and 22, arrows indicate translocation of p-Erk-1/2 kinase into nucleus.

As shown in Fig. 21, in young cells, at 5 min after treatment, p-Erk-1/2 was strongly observed in cytoplasm,

at 30 min after treatment, p-Erk-1/2 translocated into nucleus was seen and at 60 min after treatment, p-Erk-1/2 was weakly observed only in cytoplasm. These results indicate that Erk-1/2 is activated (phosphorylated) within 5 min after treatment, the resulted p-Erk-1/2 is translocated into nucleus to regulate transcription of several genes at 30 min and is finally inactivated at 60 min. In contrary to the young cells, old cells exhibited that p-Erk-1/2 was strongly observed in cytoplasm irrespective of EGF treatment, which is also found in the results of western blotting. Interestingly, old cells showed no p-Erk-1/2 translocated into nucleus.

Moreover, as shown in Fig. 22, old cells treated with sense oligonucleotide showed no p-Erk-1/2 in nucleus and vice versa for old cells treated with antisense oligonucleotide.

These results demonstrate that the inhibition of the expression of caveolin-1 with antisense oligonucleotide is responsible for the restoration of Erk-mediated signal cascade. Furthermore, the results indicate that caveolin-1 is also involved in the restoration of translocation into nucleus, which is generally blocked in old cells.

Example XX: Methylation of CpG Island of Caveolin-1 Gene

It is well known that upon aging, the expression of p16/Ink4a is increased with the decrease of the

methylation level of CpG island located in promoter thereof (Jarrard DF., *Cancer Res.*, 15;59(12):2957-2964(1999)). Furthermore, it has been revealed that caveolin-1 also has a similar methylation pattern in cancer cell (Cui J., *Prostate*, 15;46(3):249-256(2001)). Therefore, the inventors examined whether the decreased expression of caveolin-1 is ascribed to such methylation.

Young fibroblasts (PDL 20) were treated with 1 μM demethylating agent, 5-aza-deoxycytidine (Sigma) in DMSO and then periodically treated with the agent at the time of changing media for 2-3 weeks. The induction of cellular senescence was verified by SA β-gal activity staining as Example III. To investigate the expression of related proteins, the treated cells were harvested in different days and western blotting was performed as previously described. In western blotting, anti-p53 antibody, anti-p16 antibody, anti-caveolin-1 antibody and anti-actin antibody were purchased from Santa Cruz Biotechnology, Inc.

As shown in Fig. 23a representing the results of SA β-gal activity staining, the young cells treated for about 2 weeks showed senescent cell-like phenomenon. As shown in Fig. 23b representing the results of western blotting, with demethylation, the expression of p16 and caveolin-1 were increased and the expression of p53 was not altered. Interestingly, the increased level of p16 was detected at the early phase of cellular senescence, whereas the increased level of caveolin-1 was detected earlier than

p16. These results elucidate that the increased level of caveolin-1 in senescent cells was not leaded directly by either cellular senescence or the increased level of p16 but by demethylation of CpG island in promoter of caveolin-1 gene.

As a result, it is revealed that cellular senescence can be modulated by the methylation level of the promoter, in particular, CpG island of caveolin-1 gene.

Having described a preferred embodiment of the present invention, it is to be understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in this art, and the scope of this invention is to be determined by appended claims and their equivalents.

What is claimed is:

1. A method for detecting a senescent cell comprising determining the amount of a protein involved in cellular senescence, wherein the protein is one or more selected from the group consisting of amphiphysin protein and caveolin protein.

2. The method according to claim 1, wherein the cell is derived from mammalian cell.

3. The method according to claim 1, wherein the amphiphysin protein is amphiphysin-1 and the caveolin protein is caveolin-1 protein.

4. The method according to claim 1, wherein the method is performed by western blotting method.

5. A method for detecting a senescent cell comprising determining the amount of a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is one or more selected from the group consisting of amphiphysin protein and caveolin protein.

6. The method according to claim 5, wherein the cell is derived from mammalian cell.

7. The method according to claim 5, wherein the

polynucleotide encodes amphiphysin-1 or caveolin-1 protein.

8. The method according to claim 5, wherein the polynucletide is selected from the group consisting of gDNA, cDNA and mRNA.

9. A composition for modulating cellular senescence comprising the effective amount of a protein involved in cellular senescence of a cell, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

10. A composition for modulating cellular senescence comprising the effective amount of a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

11. A composition for modulating cellular senescence comprising the effective amount of an antisense oligonucleotide which hybridizes to a polynucleotide encoding a protein involved in cellular senescence and thereby inhibits the polynucleotide from expressing the protein, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

12. A composition for modulating cellular senescence

comprising the effective amount of a methylating agent or a demethylating agent, in which the agent methylates or demethylates bases of a polynucleotide encoding caveolin protein.

13. The composition according to any one of claims 9 to 12, wherein the cell is derived from mammalian cell.

14. The composition according to any one of claims 9 to 12, wherein the protein involved in cellular senescence is amphiphysin-1 protein or caveolin-1 protein.

15. The composition according to claim 10, wherein the polynucleotide is gDNA or cDNA.

16. The composition according to claim 10, wherein the polynucleotide encoding a protein involved in cellular senescence is contained in expression vector for eucaryotic cell.

17. The composition according to claim 11, wherein the protein involved in cellular senescence is caveolin protein.

18. The composition according to claim 17, wherein the antisense oligonucleotide hybridizes to translational initiation region of caveolin mRNA.

19. The composition according to claim 12, wherein the methylating agent is selected from the group consisting of methylazoxymethanol acetate, Temozolomide and N-methyl-N-nitrosourea.

20. The composition according to claim 12, wherein the demethylating agent is selected from the group consisting of 5-aza-deoxycytidine, 5-azacytidine, 6-azacytidine and 8-azaguanine.

21. The composition according claim 19 or 20, wherein the agent methylates or demethylates CpG island from promoter of the polynucleotide encoding caveolin-1 protein.

22. A composition for modulating cellular senescence comprising the effective amount of dominant negative amphiphysin-1 gene.

23. The composition according to claim 22, wherein the dominant negative amphiphysin-1 gene is a polynucleotide encoding a polypeptide comprising the amino acid sequence 250 to 588 represented by SEQ ID NO:2.

24. A method for modulating cellular senescence in a patient in need thereof, comprising administering to the patient the effective amount of a protein involved in

cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

25. A method for modulating cellular senescence in a patient in need thereof, comprising administering to the patient the effective amount of a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

26. A method for modulating cellular senescence in a patient in need thereof, comprising administering to the patient the effective amount of an antisense oligonucleotide which hybridizes to a polynucleotide encoding a protein involved in cellular senescence and thereby inhibits the polynucleotide from expressing the protein, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

27. A method for modulating cellular senescence in a patient in need thereof, comprising administering to the patient the effective amount of a methylating agent or a demethylating agent, in which the agent methylates or demethylates bases of a polynucleotide encoding caveolin protein.

28. The method according to any one of claims 24 to 27, wherein the cell is derived from mammalian cell.

29. The method according to any one of claims 24 to 27, wherein the protein involved in cellular senescence is amphiphysin-1 protein or caveolin-1 protein.

30. The method according to claim 25, wherein the polynucleotide is gDNA or cDNA.

31. The method according to claim 25, wherein the polynucleotide encoding a protein involved in cellular senescence is contained in expression vector for eucaryotic cell.

32. The method according to claim 26, wherein the protein involved in cellular senescence is caveolin protein.

33. The method according to claim 32, wherein the antisense oligonucleotide hybridizes to translational initiation region of caveolin mRNA.

34. The method according to claim 27, wherein the methylating agent is selected from the group consisting of methylazoxymethanol acetate, Temozolomide and N-methyl-N-nitrosourea.

35. The method according to claim 27, wherein the demethylating agent is selected from the group consisting of 5-aza-deoxycytidine, 5-azacytidine, 6-azacytidine and 8-azaguanine.

36. The method according claim 34 or 35, wherein the agent methylates or demethylates CpG island from promoter of the polynucleotide encoding the caveolin protein.

37. A method for identifying a substance affecting the senescence of a cell, which comprises the steps of:

(a) culturing the cell in the presence of the substance to be tested;

(b) isolating a protein from the cell;

(c) contacting the isolated protein with an antibody specific to a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein; and

(d) determining the amount of the isolated protein bound to the antibody.

38. A method for identifying a substance affecting the senescence of a cell, which comprises the steps of:

(a) culturing the cell in the presence of the substance to be tested;

(b) isolating RNA from the cell;

(c) contacting the isolated RNA with a probe derived

from a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein; and

(d) determining the amount of the isolated RNA hybridized to the polynucleotide encoding a protein involved in cellular senescence.

39. The method according to claim 37 or 38, wherein the cell is derived from mammalian cell.

40. The method according to claim 37 or 38, wherein the protein is amphiphysin-1 protein or caveolin-1 protein.

41. A kit for detecting a senescent cell comprising a probe derived from a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

42. The kit according to claim 41, wherein the cell is derived from mammalian cell.

43. The kit according to claim 41, wherein the protein is amphiphysin-1 protein or caveolin-1 protein.

44. The kit according to claim 41, wherein the probe is

immobilized on a solid support.

45. The kit according to any one of claims 41 to 44, further comprising a label for detecting the presence of the probe.

46. A biomarker for identifying cellular senescence comprising a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

47. A biomarker for identifying cellular senescence comprising a polynucleotide encoding a protein involved in cellular senescence, wherein the protein is selected from the group consisting of amphiphysin protein and caveolin protein.

48. The biomarker according to claim 46 or 47, wherein the protein is amphiphysin-1 or caveolin-1 protein.

FIGURE

Fig. 1

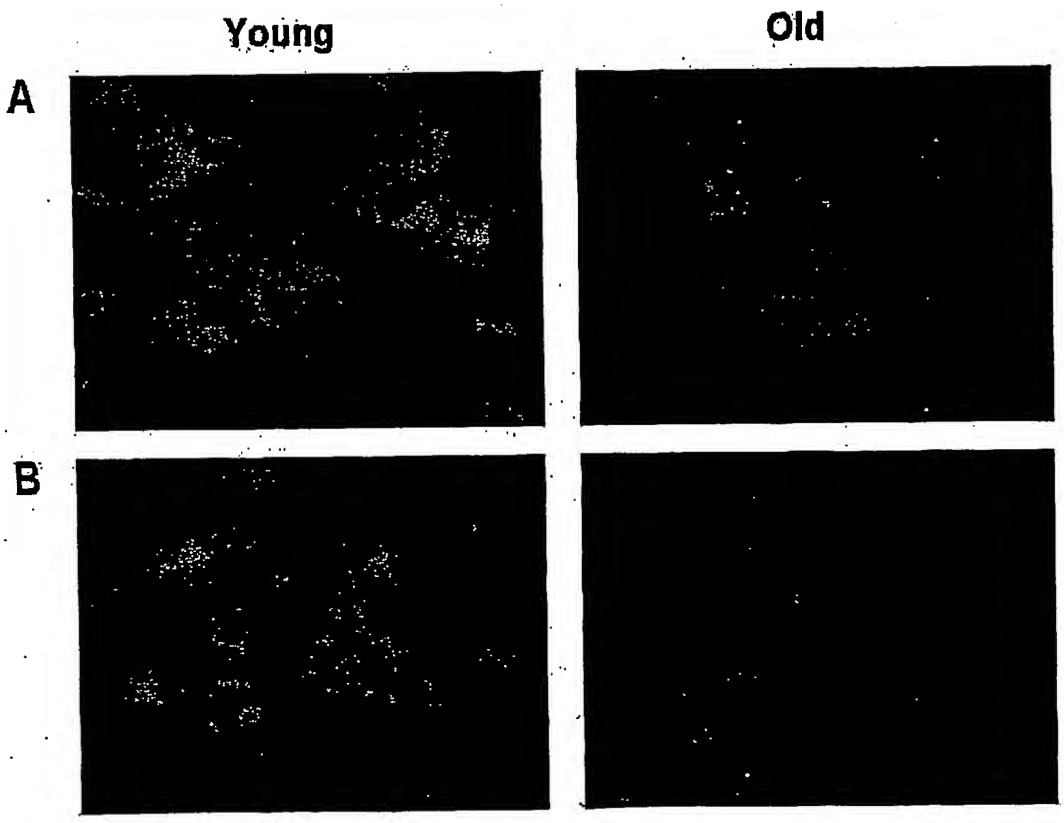


Fig. 2

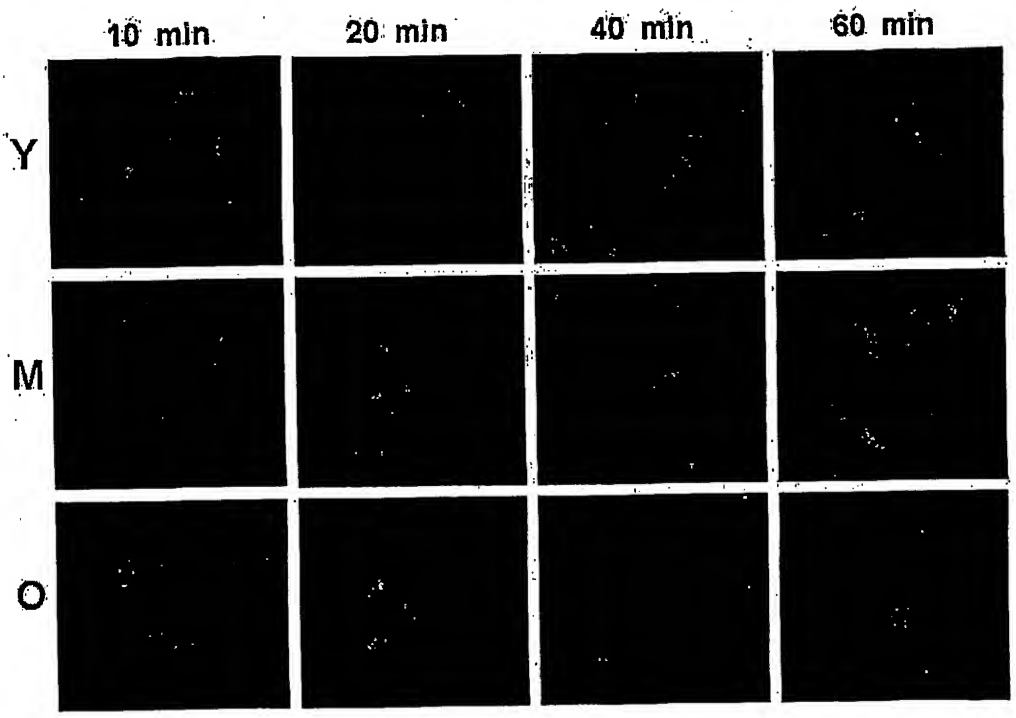


Fig. 3

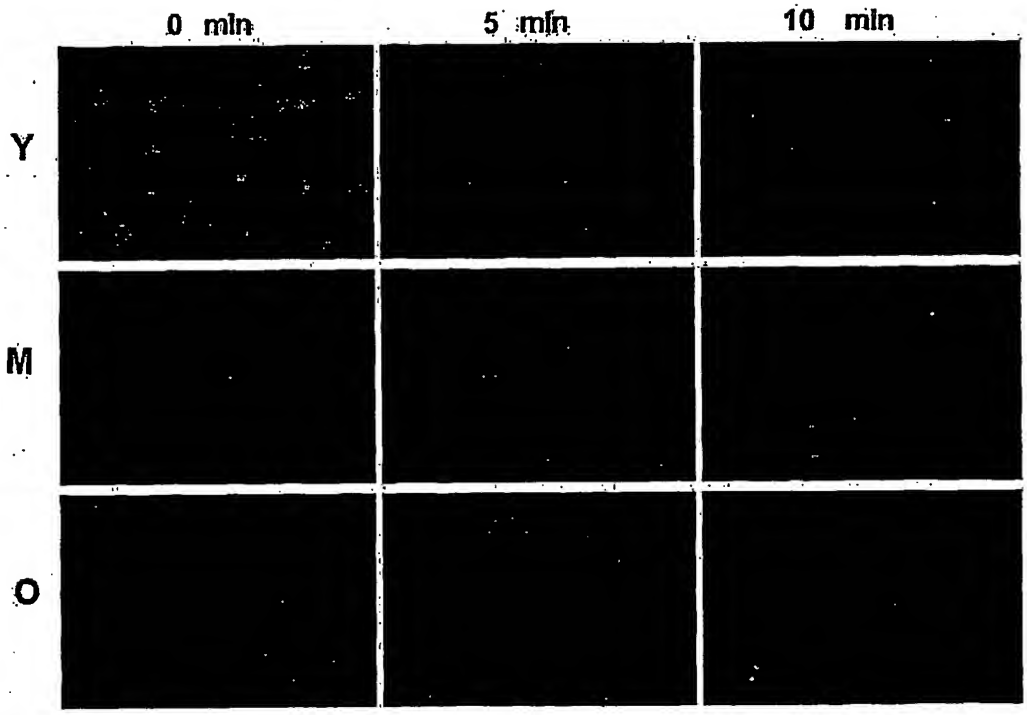


Fig. 4

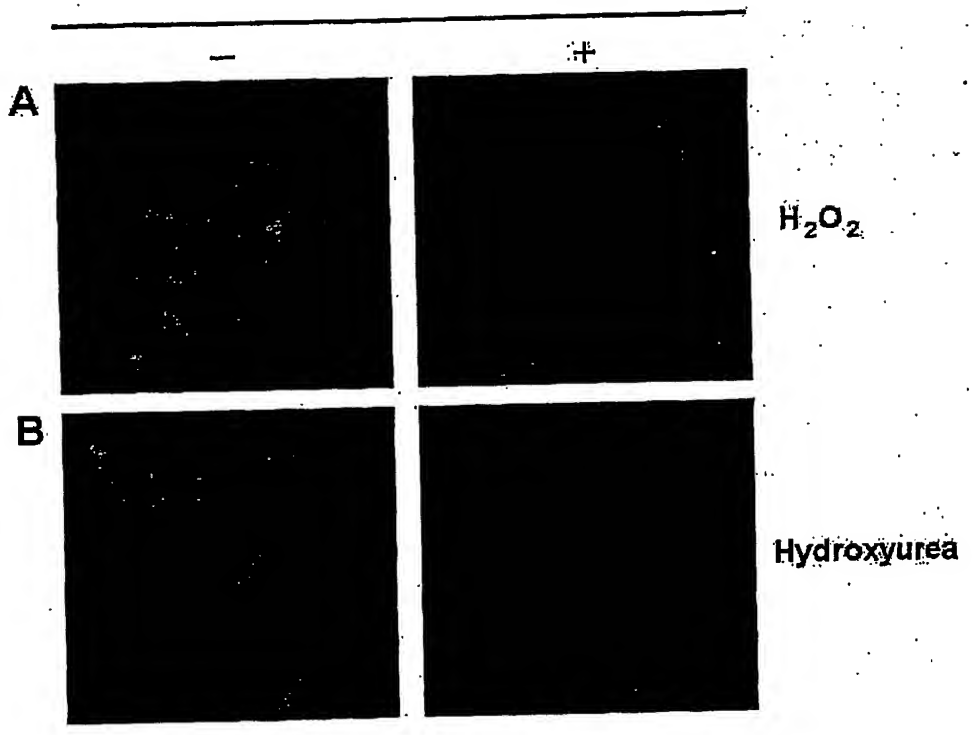


Fig. 5

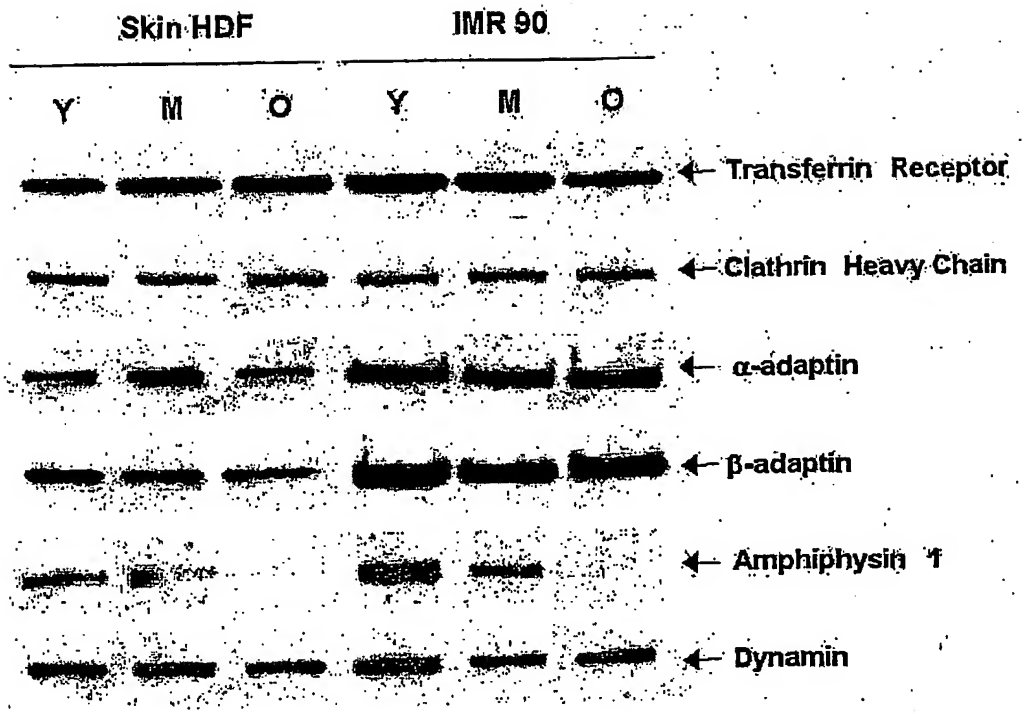
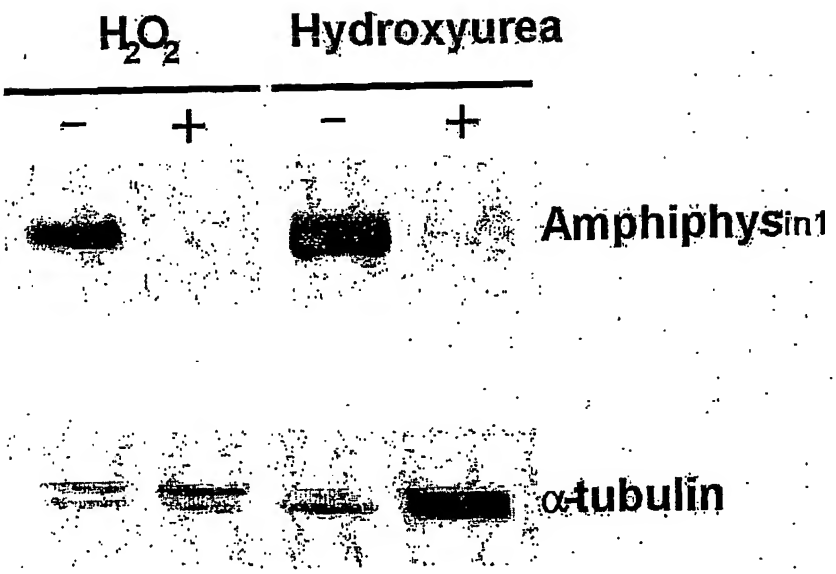

Skin HDF
IMR 90
Y
M
O
Y
M
O
Transferrin Receptor
Clathrin Heavy Chain
α-adaptin
β-adaptin
Amphiphysin I
Dynamin

Fig. 6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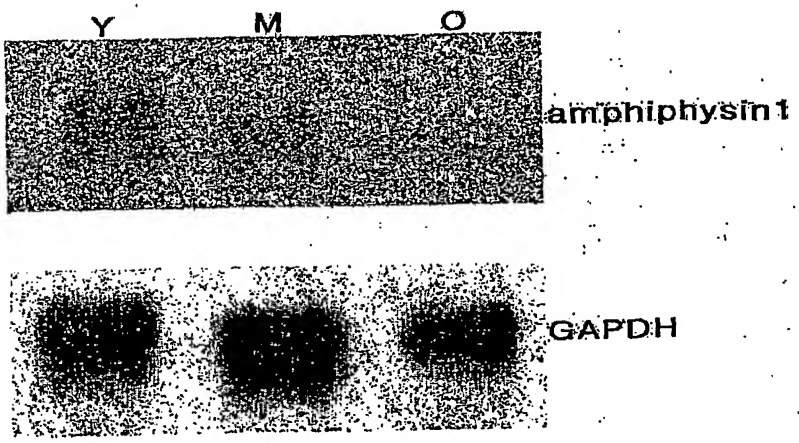
−
+
−
+
Amphiphysin1
α-tubulin

Fig. 7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M
O
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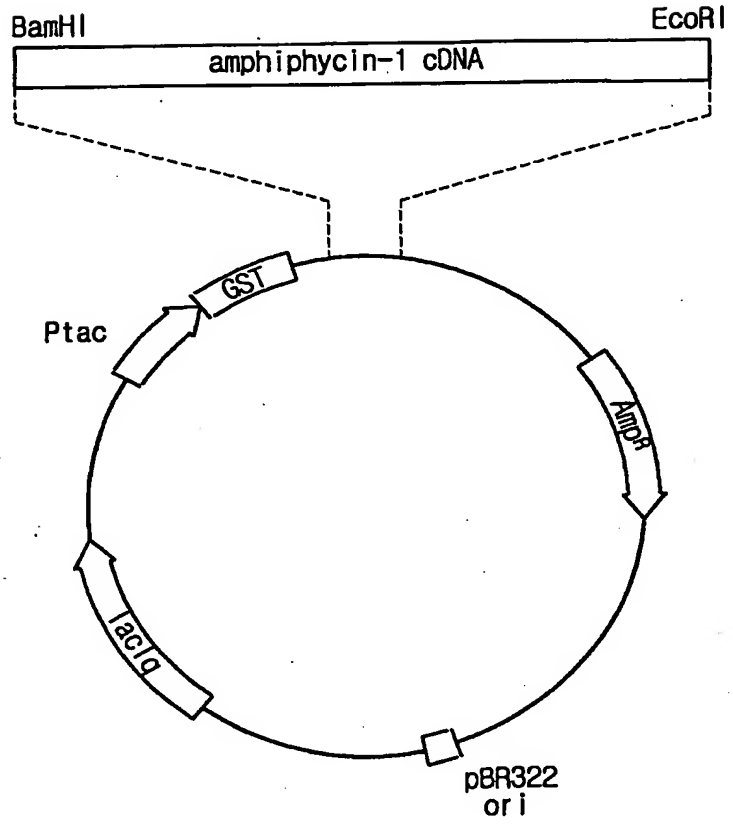
GAPDH

Fig. 8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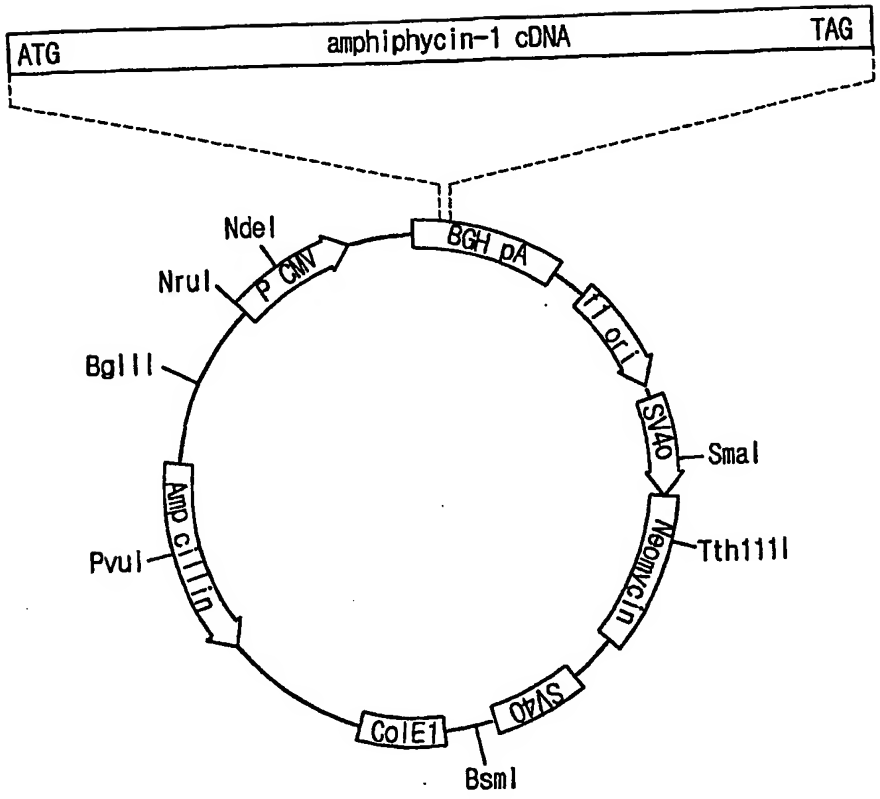
ori

Fig. 9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TAG
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NruI
BglII
PvuI
P CMV
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Tth111I
SV40
ColE1
BsmI
Amp cITIin

Fig. 10

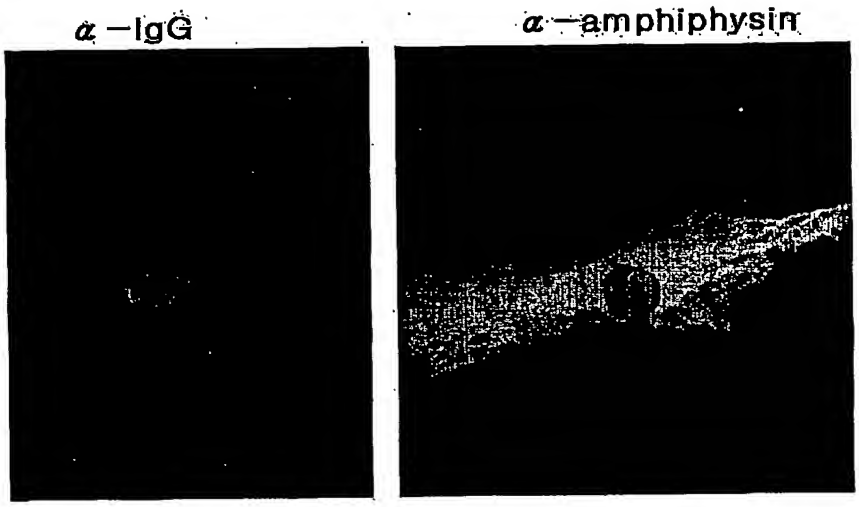
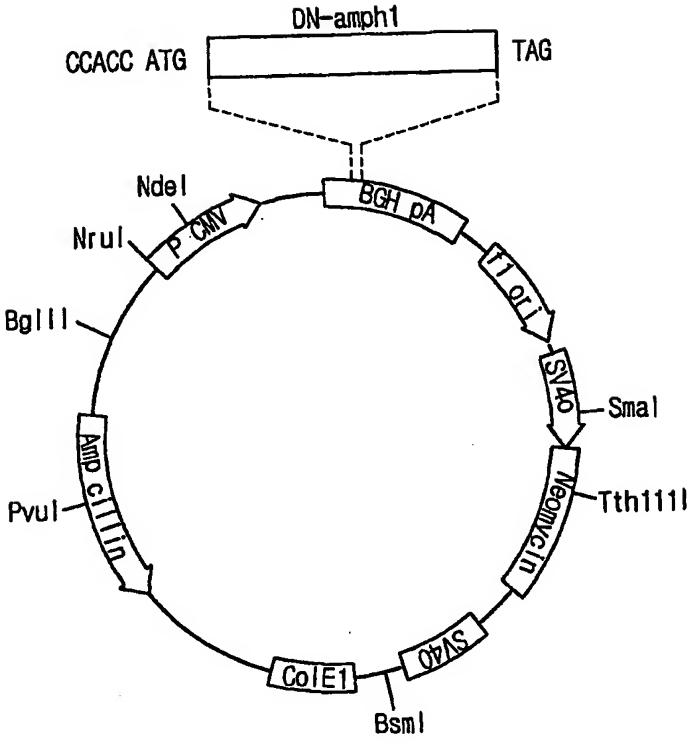


Fig. 11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NruI
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PvuI
BsmI
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SV40
SmaI
NeoMycin
Tth111I
SV40
ColE1
Amp cIIIin

Fig. 12

Fig. 13a

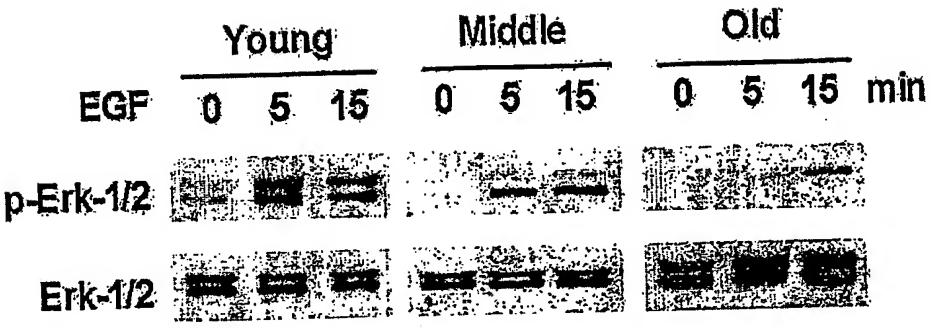


Fig. 13b

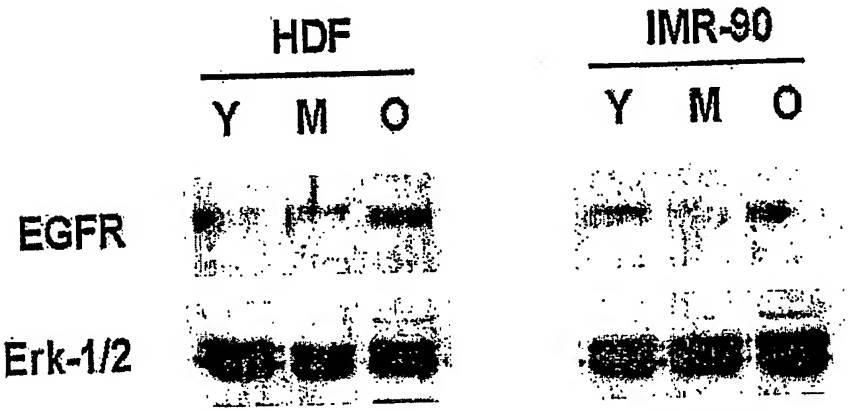


Fig. 14

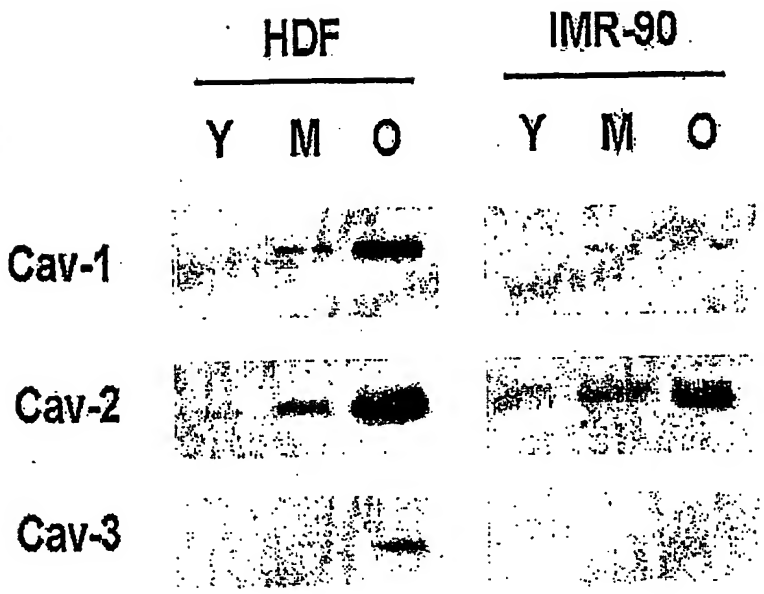


Fig. 15

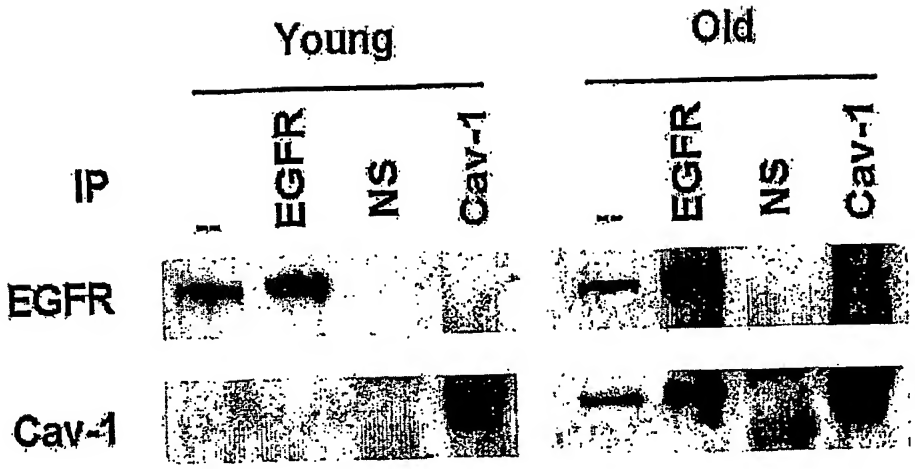


Fig. 16

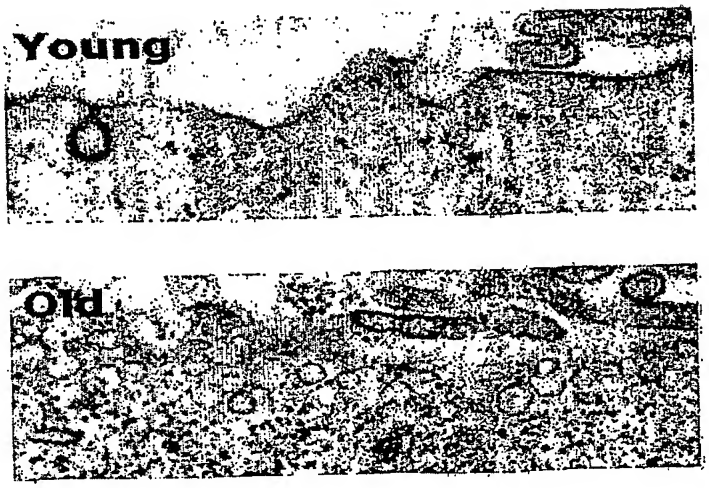


Fig. 17

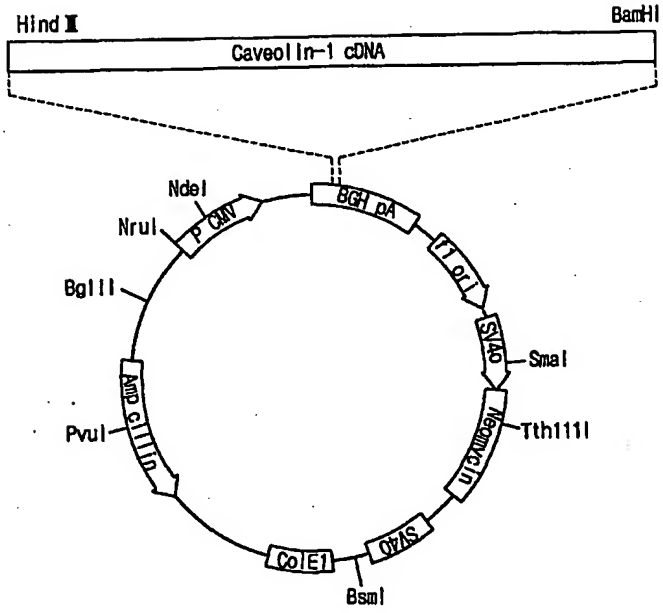
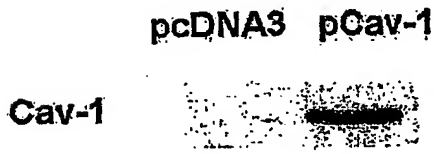


Fig. 18

A



B

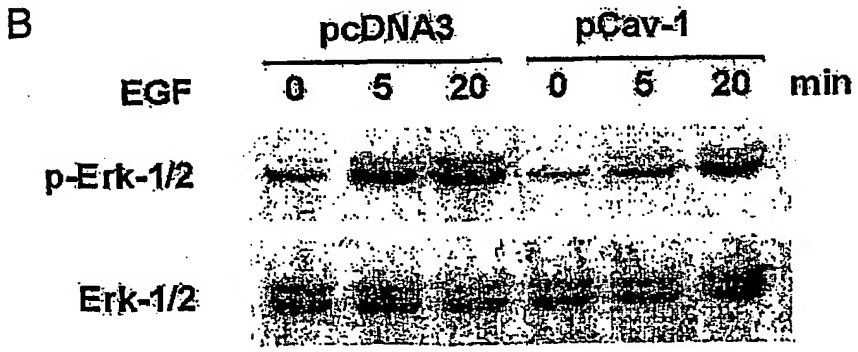


Fig. 19

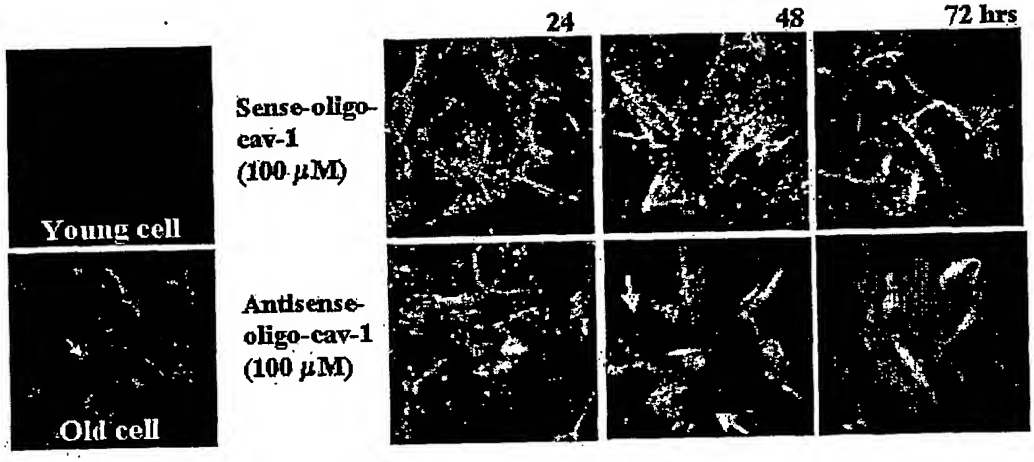

24
48
72 hrs
Young cell
Old cell
Sense-oligo-cav-1 (100 μM)
Antisense-oligo-cav-1 (100 μM)

Fig. 20

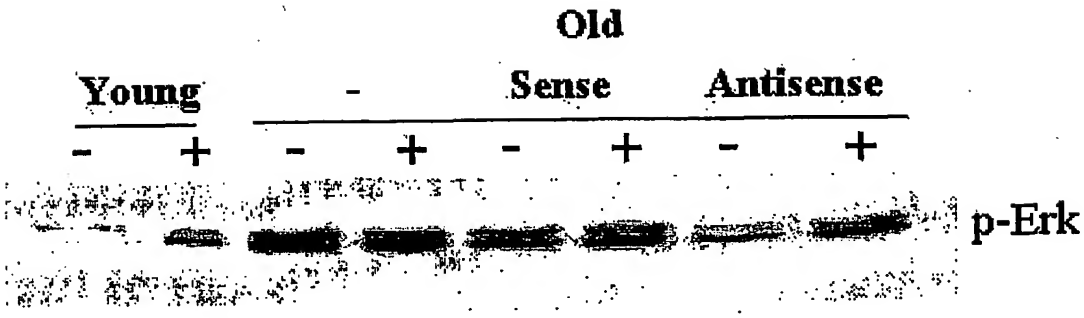


Fig. 21

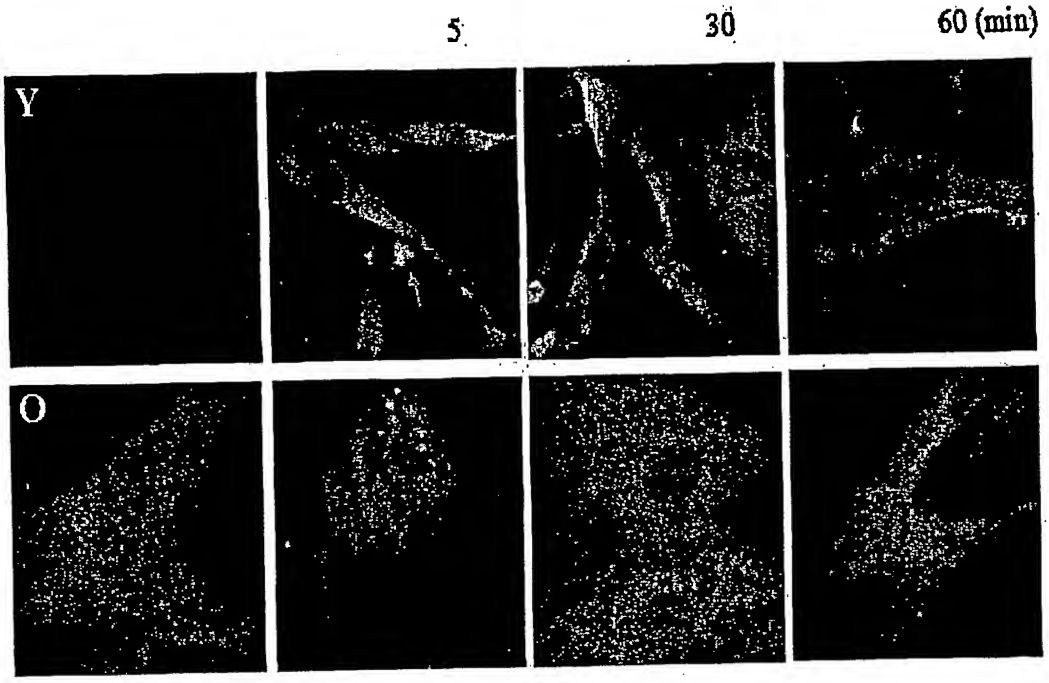


Fig. 22

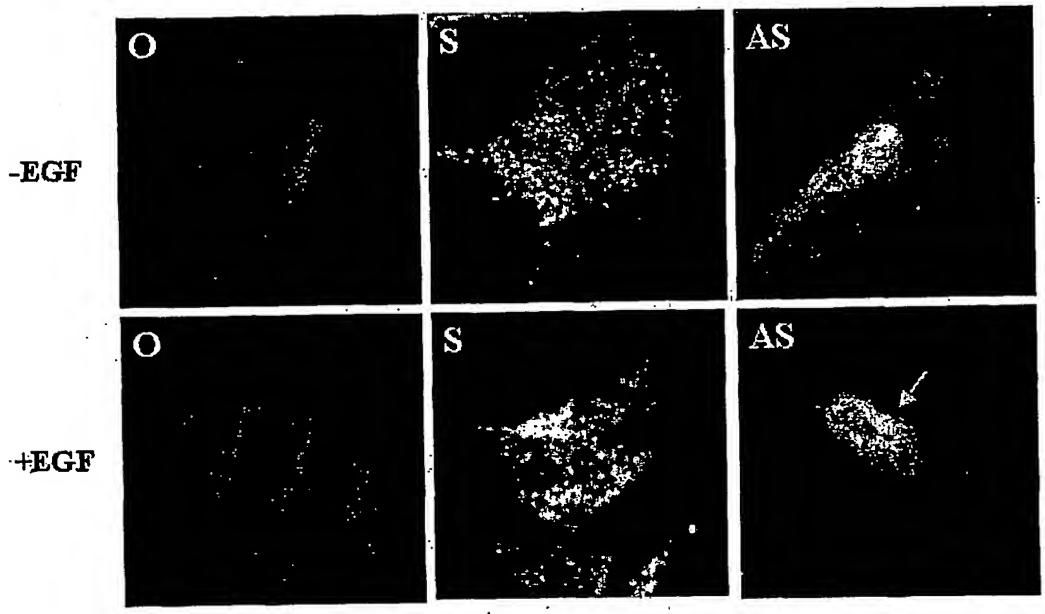


Fig. 23a

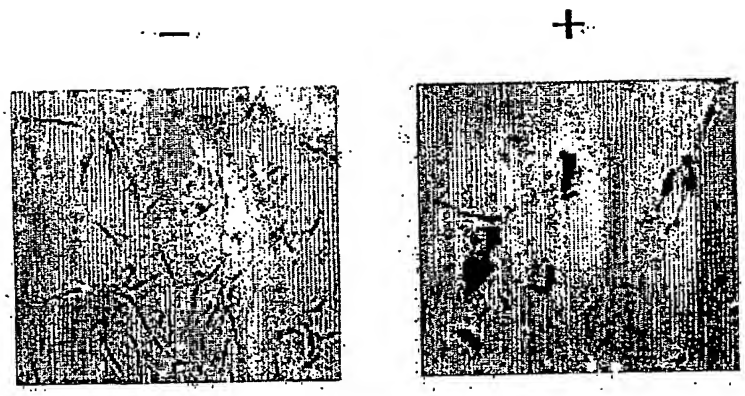
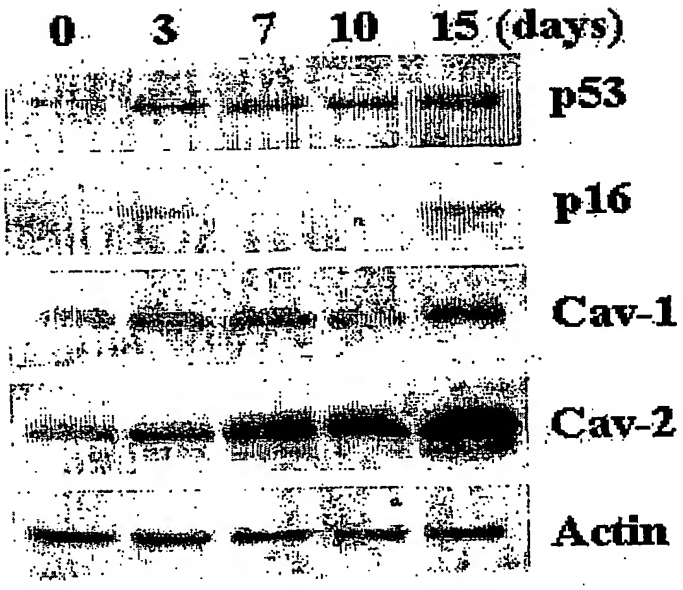


Fig. 23b



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1

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Ser Arg Val Tyr Ser Ile Tyr Val His Thr Val Cys Asp Pro Leu Phe
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Glu Ala Val Gly Lys Ile Phe Ser Asn Val Arg Ile Asn Leu Gln Lys
165 170 175

Glu Ile

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 01/01159

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: G01N 33/68, 33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

AT-Patent documents

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REGISTRY and CA Databases, STN-International, INTERNET - MEDLINE Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W.-Y- Park et al., "Up-regulation of Caveolin Attenuates Epidermal Growth Factor Signaling in Senescent Cells", J. Biol. Chem., Vol. 275 (27), 7 July 2000 (07.07.00) , pages 20847-20852 *see the whole document*	4,9,13,14,24, 28,29,37,39,40
A	*see the whole document*	46,48

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 October 2001 (19.10.2001)	6 December 2001 (06.12.2001)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer WENIGER Telephone No. 1/53424/341

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 01/01159

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-3
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The method according to claims 1-3 is only characterized by its aim and the problem to be solved and not by technical features concerning the essential steps by which a process or a method should be characterized.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Fist of all, the present application comprises the two totally different subject matters as follows:

I: Claims 1-48, partly, as far as directed to the detection, the modulation, etc. of senescent cells with the use of a caveolin protein or a poly- or oligonucleotide thereof.

II: Claims 1-11, 22-26, 30-33 and 37-48, as far as directed to the detection, the modulation, etc. of senescent cells with the use of an amphiphysin protein or a poly- or oligonucleotide thereof.

These two subject matters do not share any common features, because for the following reasons the proteins caveolin and amphiphysin neither have anything in common nor are functionally related or at least linked:

- Caveolin is an integral membrane protein, functions as a scaffoding protein withing the caveolae membrane and interacts with signalling proteins, namely EGFR, G-proteins, Src-like kinases, Ha-Ras, protein kinase C and so on. In senescent cells increased levels of caveolin proteins can be found which result in the suppression of the activation of EGFR upon EGF stimulation.
- - Amphiphysin is a synaptic vesicle associated protein which binds to multiple vesicle recycling proteins including dynamin, adaptin, clathrin and synaptojanin, implicating amphiphysin in the process of synaptic endocytosis. Senescence of cells is accompanied with an decrease of amphiphysin-1 which results in reduced receptor-mediated endocytosis in human diploid fibroplasts.

Subject matter I:
For the search only those parts of the claims have been taken into consideration which belong to subject matter I, but these claims (at least partly) belong to five groups of inventions, which are not linked as to form a single inventive concept:

Group I: claims 1-4, 37, 39, 40, 46 and 48, directed to a method for detecting a senescent cell or for identifying a substance affecting the senescence of a cell comprising determining the amount of a caveolin protein and a kit and a biomarker therefore.

Group II: claims 5-8, 38-45, 47 and 48, directed to a method for detecting a senescent cell or for identifying a substance affecting the senescence of a cell comprising determining the amount of a polynucleotide encoding a caveolin protein and a kit and a biomarker therefore.

Group III: claims 9, 13, 14, 24, 28 and 29, directed to a composition for modulating cellular senescence comprising the effective amount of a caveolin protein and a method for modulating cellular senescence with such a composition.

Group IV: claims 10, 11, 13-18, 25, 26, 28-33, directed to a composition for modulating cellular senescence comprising the effective amount of a polynucleotide encoding a caveolin protein or an oligonucleotide which hybridises to such a polynucleotide and a method for modulating cellular senescence with such a composition.

Group V: claims 12-14, 19-21, 27-29, 34-36 directed to a composition for modulating cellular senescence comprising the effective amount of a methylating agent or a demethylating agent methylating a polynucleotide encoding .

As Groups I and III (except claims 1-3) could be searched without effort justifying an additional fee, these two groups have been searched without invitation to pay an additional fee for group III of inventions.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 01/01159

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W. A -Y- PARKETAL ., "UP		none.	